BMP4 mutations in tooth agenesis and low bone mass

Miao Yu[a], Hao Wang[a], Zhuangzhuang Fan[a], Chencheng Xie[b], Haochen Liu[a], Yang Liu[a,**], Dong Han[a,**], Sing-Wai Wong[c,*], Hailan Feng[a]

[a] *Department of Prosthodontics, Peking University School and Hospital of Stomatology, National Clinical Research Center for Oral Diseases, National Engineering Laboratory for Digital and Material Technology of Stomatology, Beijing Key Laboratory of Digital Stomatology, China*
[b] *Department of Internal Medicine, Sanford Medical School, University of South Dakota, Sioux Falls, SD, 57105, USA*
[c] *Department of Periodontology, Adams School of Dentistry, University of North Carolina at Chapel Hill, Chapel Hill, NC, 27599, USA*

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ABSTRACT

Objective: To identify an uncommon genetic cause of tooth agenesis (TA) by utilizing whole exome sequencing (WES) and targeted Sanger sequencing in a cohort of 120 patients with isolated TA.
Design: One deleterious mutation in the gene encoding bone morphogenetic protein 4 (BMP4) was identified in 6 unrelated patients with TA by WES. After that, the coding exons of *BMP4* were examined in 114 TA patients using Sanger sequencing. Dual-energy X-ray absorptiometry (DEXA) was used to measure the bone mineral density of patients who carried a *BMP4* mutation. Finally, preliminary functional studies of two BMP4 mutants were performed.
Results: We detected 3 novel missense mutations (c.58 G > A: p.Gly20Ser, c.326 G > T: p.Arg109Leu and c.614 T > C: p.Val205Ala) and 1 reported mutation in the *BMP4* gene among 120 TA probands. The previously reported *BMP4* mutation (c.751C > T: p.His251Tyr) was associated with urethra and eye anomalies. By extending the pedigrees, we determined that the tooth phenotypes had an autosomal dominant inheritance pattern, as individuals carrying a *BMP4* mutation exhibit different types of dental anomalies. Interestingly, we observed that patients harboring a *BMP4* mutation manifested early onset osteopenia or osteoporosis. Further *in vitro* functional assays demonstrated that two BMP4 mutants resulted in a decreased activation of Smad signaling. Therefore, a loss-of-function in BMP4 may contribute to the clinical phenotypes seen in this study.
Conclusions: We identified 4 mutations in the *BMP4* gene in 120 TA patients. To our knowledge, this is the first study to describe human skeletal diseases associated with *BMP4* mutations.

1. Introduction

Tooth agenesis is one of the most prevalent developmental anomalies in humans and can adversely affect oral functions and esthetics (Nieminen, 2009). Current estimates of the prevalence of tooth agenesis ranges from 2.6% to 11.3%, when third molars are excluded (De Coster, Marks, Martens, & Huysseune, 2009). Usually, tooth agenesis indicates the most common form of non-syndromic tooth agenesis, where abnormalities only occur in the dental tissue (Shimizu & Maeda, 2009; M. Yu, Wong, Han, & Cai, 2019). Based on the number of missing teeth (excluding the third molars), tooth agenesis can be categorized as hypodontia (missing fewer than six teeth), oligodontia (missing six or more teeth) and anodontia (missing the entire dentition). Along with a

reduction of their number of teeth, patients with tooth agenesis may also have alterations in tooth size, shape or structure in their remaining teeth (Wong et al., 2018). Multiple lines of evidence have demonstrated that genetic factors play a predominant role in the aetiopathogenesis of dental agenesis (Brook, 2009; Williams & Letra, 2018). We and others have reported a cluster of common causative genes for tooth agenesis that include *MSX1* (Vastardis, Karimbux, Guthua, Seidman, & Seidman, 1996; Wong, Liu, Han et al., 2014), *PAX9* (Stockton, Das, Goldenberg, D'Souza, & Patel, 2000; Wong et al., 2018), *AXIN2* (Lammi et al., 2004; Wong, Liu, Bai et al., 2014), *EDA* (Han et al., 2008), *WNT10A* (Song et al., 2014; van den Boogaard et al., 2012), *LRP6* (Dinckan, Du, Petty et al., 2018; Massink et al., 2015), and *WNT10B* (Kantaputra et al., 2018; Yu et al., 2016). These mutations account for more than 90% of

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human TA causative mutations (Yu et al., 2019). However, other TA-associated genes, such as *BMP4* (Huang et al., 2013), *GREM2* (Kantaputra et al., 2015), *ANTXR1* (Dinckan, Du, Akdemir et al., 2018), and *IKBKG* (Sun et al., 2019) have been rarely studied, and thus require further investigation into identifying if they play a role in TA (D'Souza et al., 2013).

Bone morphogenetic protein 4 (BMP4) is a member of the transforming growth factor-beta (TGF-β) superfamily of secretory molecules that are involved in the BMP signaling pathway (Bostrom, Blazquez-Medela, & Jumabay, 2019). The BMP pathway has been shown to play multiple roles in tooth development, cell differentiation and bone formation (Maas & Bei, 1997; Salazar, Gamer, & Rosen, 2016; Vainio, Karavanova, Jowett, & Thesleff, 1993). During early tooth development, the expression of Bmp4 shifts from the dental epithelium to the mesenchyme, by which Bmp4 plays a central role in epithelial-mesenchymal interactions during dental morphogenesis (Vainio et al., 1993; Zhang, Chen, Song, Liu, & Chen, 2005). *Bmp4* conditional knockout mice exhibit varying degrees of severity of dental phenotypes, including molar agenesis, and reduced tooth size (Jia et al., 2013). In another mouse model, mice knocked out for both *Bmp4* and *Bmp2* display a severe defect in skeletal development (Bandyopadhyay et al., 2006). Furthermore, the transcription of *Bmp4* remain highly activated during postnatal skeletal remodeling after bone maturation (Pregizer & Mortlock, 2015), suggesting that *Bmp4* is essential for bone development and homeostasis.

Since Bmp4 plays an important role in dental morphogenesis (Jia et al., 2013) and a heterozygous missense *BMP4* mutation has been reported in a family with tooth agenesis (Huang et al., 2013), we hypothesized that *BMP4* could be a reliable candidate gene for tooth agenesis. In this study, we report the discovery of a missense mutation in *BMP4* by WES in a core family with TA. Through Sanger sequencing of the *BMP4* gene, 3 additional *BMP4* mutations were identified among a cohort of TA patients. Moreover, we discovered that patients containing a *BMP4* mutation were affected with early onset osteopenia or osteoporosis. Overall, this study identified novel genetic mutations in *BMP4* among TA patients, and characterized a novel phenotype associated with *BMP4* mutations.

2. Materials and methods

2.1. Studied patients

A cohort of 120 patients with non-syndromic TA (missing teeth ≥ 6) was recruited from the Department of Prosthodontics in the Peking University School and Hospital of Stomatology (PKUSS), Beijing, China. The missing permanent tooth number did not include the third molar. To verify the number and pattern of missing teeth, intra-oral examinations and panoramic radiographs were performed on patients. These patients confirmed that their missing permanent teeth were not due to injuries or extractions. Healthy controls (n = 100) were recruited from the PKUSS orthodontic clinic. Patients or their patients provided written informed consent for the genetic analyses and radiographs used in this study. This study was approved by the Ethics Committee of PKUSS.

2.2. DNA sequencing

Genomic DNA was isolated from each patient's peripheral blood lymphocytes using BioTek DNA Whole-blood Mini Kit (BioTek, Beijing, China) or from saliva using Oragene tubes (DNA Genotek, Canada). Whole exome sequencing (WES) was performed by the Beijing Genomic Institute (BGI, Beijing, China) using DNA from lymphocytes from 6 non-consanguineous individuals with tooth agenesis. The coding exons of the *BMP4* gene of all patients were amplified by polymerase chain reaction (PCR) using the following primer sequences: exon 3-F (5′− CCATCTTGCCCCTCCATTTCTA-3′); exon 3-R (5′-CTTCTTCCCCAGGGC

TTTCACT-3′); exon 4a-F (5′- TGCTTATTTTCCCCCAGTAGGT-3′); exon 4a-R (5′−CCCGCTGTGAGTGATGCTT-3′); exon 4b-F (5′-GGGCCAGCA TGTCAGGATTAGC-3′) and exon 4b-R (5′- GTGGGTGAGTGGATGGGA ACG-3′). The amplified PCR products were sequenced by Tsingke Biological (Beijing, China) and the results were blasted on NCBI.

2.3. Dual-energy X-ray absorptiometry (DEXA)

Three patients took part in the dual-energy X-ray absorptiometry (DEXA) examination. The bone mineral density (BMD) of their lumber spine (L1-L4) and right hip bone was measured using a Hologic Discovery A bone densitometer (Hologic, Boston, MA, USA) at the Peking University Third Hospital, Beijing, China.

2.4. Conservation analysis

The alignment analysis of the BMP4 amino acid sequence among multiple species (NP_001193.2) was conducted using ClustalX 2.1. The BMP4 amino acid sequences of different vertebrate species were obtained from NCBI.

2.5. BMP4 plasmid construction and site-directed mutagenesis

The plasmids used in this study were constructed by the BGI. The full-length coding sequence of wild type (WT) BMP4 (accession number: NM_001202.6) was sub-cloned into the HindIII and EcoRI restriction enzyme sites of the pEGFP-C1 vector to generate a pEGFP-C1-BMP4-WT plasmid. *In vitro* site-directed mutagenesis was performed to construct the pEGFP-C1-V205A (V205A) and pEGFP-C1-H251Y (H251Y) mutant plasmids. These constructs were validated by Sanger sequencing.

2.6. Cell culture, transfection and western blot

HEK 293 T cells (provided by Prof. Yixiang Wang) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, CA, USA) and 1% penicillin/streptomycin (Life Technologies) at 37 °C in a humidified atmosphere of 5% CO_2. Cells were transiently transfected with either the WT and mutant plasmids (V205A and H251Y) using Lipo3000 (Invitrogen, CA, USA), and then collected for western blot analysis. 20 μg protein of each sample was resolved by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. Blots were probed with the following antibodies: anti-GFP (ab32146, Abcam, Cambridge, UK), phospho-Smad 1/5/9 (13820, Cell Signaling Technology, MA, USA), total Smad 1/5 (bs-2973R, Bioss Biotechnology, Beijing, China) and GAPDH (KM9002 T, Sungene Biotech, Tianjin, China). Protein densitometry was quantified using Image *J* software and normalized against the housekeeping protein, GAPDH.

2.7. Statistical analysis

Quantitative data were analyzed using one-way ANOVA with post Tukey's HSD by GraphPad Prism (GraphPad Software, San Diego, CA, USA). Data were presented as mean ± SD (n = 3) with a $p < 0.05$ considered as statistically significant.

3. Results

3.1. Mutational analysis and dental findings

WES and targeted sequencing of *BMP4* in 120 non-consanguineous TA patients revealed 5 individuals with a heterozygous *BMP4* mutation (4.12%) (Table 1), that was not seen in the 100 healthy controls. Clinical dental records of these patients were retrieved from our patient database and available panoramic radiographs (Fig. 1) were displayed.

Table 1
Summary of *BMP4* mutations present in this study.

Patient Number	Gender	Exon	Nucleotide substitution	Protein substitution	ExAC Frequency	SIFT	PolyPhen-2
#217 proband	Male	4	c.614 T > C	*p.Val205Ala*	Not present	0 (damaging)	0.912 (probably damaging)
#290 proband	Male	3	c.58 G > A	*p.Gly20Ser*	Not present	0.23 (tolerated)	0.874 (possibly damaging)
#551 proband	Female	3	c.326 G > T	*p.Arg109Leu*	2 /118184 alleles MAF:1.692e-05	0.01 (deleterious)	0.431 (benign)
#69 proband	Male	4	c.751C > T	*p.His251Tyr*	15/121170 alleles MAF:0.0001238	0.03 (deleterious)	0.974 (probably damaging)
#423 proband	Female	4	c.751C > T	*p.His251Tyr*	15/121170 alleles MAF:0.0001238	0.03 (deleterious)	0.974 (probably damaging)

Patients harboring a *BMP4* mutation exhibited variable dental phenotypes ranging from microdontia and conical teeth to severe oligodontia in their permanent dentition. The detected mutations and clinical findings are described below.

#217 proband was a 15-year-old male carrying a novel *BMP4* missense mutation *c.614 T > C* (p.Val205Ala) that was identified by WES (Fig. 2A). His eyes, facial appearance and epidermal organs (body hair, skin, nails) were normal, but he had agenesis of 16 permanent teeth, which included all his molars and some of his incisors and premolars (Fig. 1A). While proband's father exhibited bilateral microdontia in the maxillary lateral incisors, the number of teeth appeared to be normal (Table 2). No dental abnormalities were found in the proband's mother. The *BMP4 c.614 T > C* mutation of this proband was inherited from his father and resulted in the substitution of Val to Ala at residue 205. This mutation was not found in the ExAC Browser (Table 1).

A novel *BMP4* mutation (*c.58 G > A*) was detected in a 24-year old male patient (#290 proband II1) (Fig. 2B), who was congenitally missing 17 permanent teeth, which consisted of mainly the premolars and incisors (Fig. 1B and Table 2). The patient's ectodermal derived organs appeared normal, and he did not have any eye defects or a cleft palate/lip. According to the records, the patient's parents did not have any dental abnormalities and were unavailable for genetic testing. The *c.58 G > A* mutation in *BMP4* led to a substitution of Gly to Ser at residue 20, and was absent in the ExAC Browser (Table 1).

Another novel *BMP4* mutation, *c.326 G > T* (p.Arg109Leu), was identified in a 9-year-old girl (#551 proband II1) (Fig. 2C), who was affected with severe oligodontia (missing tooth number = 13). She had agenesis of all premolars, and her incisors and molars were partially affected (Table 2). Similarly, the proband's father suffered from tooth agenesis (missing tooth number = 9), while her mother was unaffected. Genetic analysis indicated that the proband's missense *BMP4* mutation

was inherited from her father (Fig. 2C). This *BMP4* variant was extremely rare - only two alleles of c.326 G > T were shown in the ExAC browser (2/118184 alleles; MAF: 1.692e-05) (Table 1).

Notably, we found that two unrelated TA patients harbored the same missense mutation in *BMP4*, *c.751C > T* (p.His251Tyr) (Fig. 2D and E). This mutation was previously reported in patients with congenital urethra and eye defects (Chen et al., 2007; X. Zhang et al., 2009). #69 proband was a 16-year-old male with mild oligodontia, and was missing 6 premolars (Fig. 1C and Table 2). He did not have any eye anomalies and denied having any urethra defects. Besides tooth agenesis, the patient also showed tooth shape anomalies – his incisors had a narrow incisal edge and the canines exhibited a slender cusp (Table 2). The patient did not have a family history of tooth agenesis, and both of his parents were unavailable for genetic screening. This variant was seen in the ExAC Browser (15/121170 alleles; MAF: 0.0001238) (Table 1).
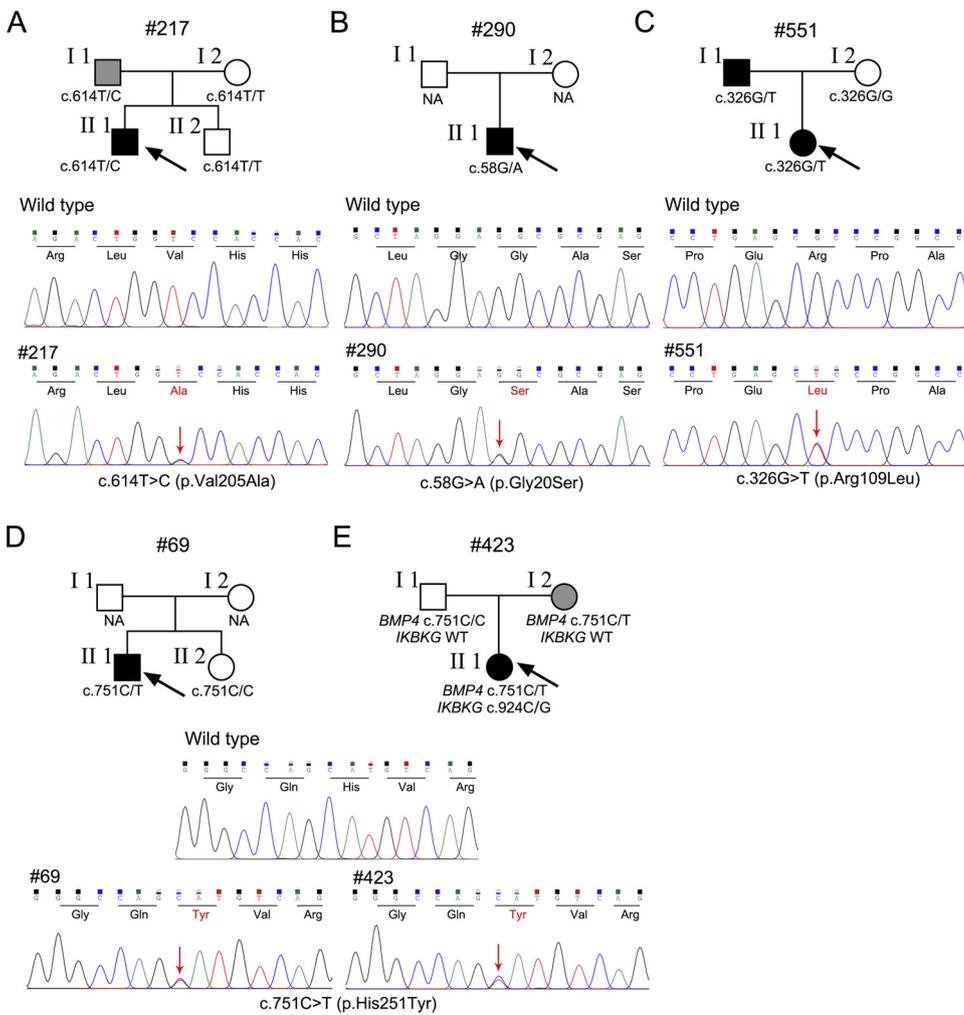
Interestingly, we segregated the *BMP4 c.751C > T* variation in our previously reported #423 proband (Fig. 2E), who carried a *de novo* nonsense mutation in *IKBKG*, a gene that causes syndromic TA (Sun et al., 2019). The patient was affected with both incontinentia pigmenti and oligodontia, but not with microphthalmia or hypospadias. Genetic analysis showed that the patient's mutant *BMP4* allele was inherited from her mother (Fig. 2E), who exhibited a mild tooth phenotype with one conical upper lateral incisor and the absence of four third molars (Table 2).

3.2. Skeletal findings

Since Bmp4 is involved in bone development and osteoblast differentiation at *in vivo* animal and *in vitro* cellular studies, we contacted patients with a *BMP4* mutation living in Beijing and the surrounding areas, and further investigations were conducted to determine if any
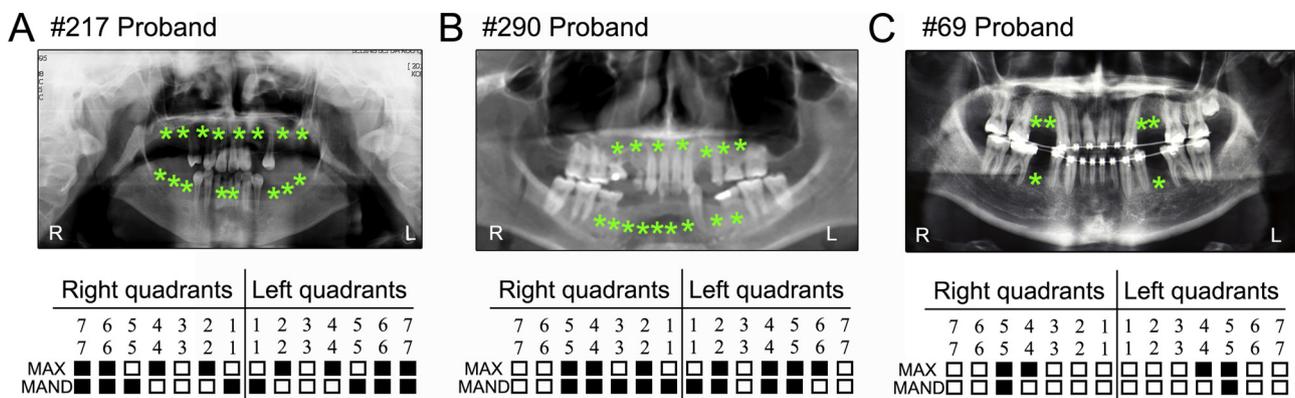
Fig. 1. Dental characteristics of patients with different *BMP4* mutations. (A) Panoramic radiograph and schematic of congenitally missing teeth of #217 proband. (B) Panoramic radiograph and schematic of congenitally missing teeth of #290 proband. (C) Panoramic radiograph and schematic of congenitally missing teeth of #69 proband. Asterisks and solid squares indicate the congenitally missing teeth. R, right; L, left; MAX, maxillary; MAND, mandibular.

A #217

I 1 (grey square) — c.614T/C   I 2 (circle) — c.614T/T
II 1 (black square, proband) — c.614T/C   II 2 (square) — c.614T/T

Wild type
Arg Leu Val His His

#217
Arg Leu Ala His His
c.614T>C (p.Val205Ala)

B #290

I 1 (square) NA   I 2 (circle) NA
II 1 (black square, proband) — c.58G/A

Wild type
Leu Gly Gly Ala Ser

#290
Leu Gly Ser Ala Ser
c.58G>A (p.Gly20Ser)

C #551

I 1 (black square) — c.326G/T   I 2 (circle) — c.326G/G
II 1 (black circle, proband) — c.326G/T

Wild type
Pro Glu Arg Pro Ala

#551
Pro Glu Leu Pro Ala
c.326G>T (p.Arg109Leu)

D #69

I 1 (square) NA   I 2 (circle) NA
II 1 (black square, proband) — c.751C/T   II 2 (circle) — c.751C/C

Wild type
Gly Gln His Val Arg

#69
Gly Gln Tyr Val Arg

E #423

I 1 (square) — BMP4 c.751C/C, IKBKG WT   I 2 (grey circle) — BMP4 c.751C/T, IKBKG WT
II 1 (black circle, proband) — BMP4 c.751C/T, IKBKG c.924C/G

#423
Gly Gln Tyr Val Arg
c.751C>T (p.His251Tyr)

Fig. 2. Pedigrees of patients with tooth agenesis and mutational analysis of human *BMP4* gene. (A–C) DNA sequencing chromatograms showing three novel heterozygous mutations of c.614 T > C, c.58 G > A and c.326 G > T in #217 proband, #290 proband and #551 proband, respectively. (D) DNA sequencing chromatograms showing a heterozygous mutation of c.751C > T in #69 proband and #423 proband. Black arrows indicate the proband on each family. Red arrows indicate point mutations. Black squares and circles represent TA patients. The grey square and circle represent individuals with tooth anomalies. "NA" indicates that a DNA sample was not available.
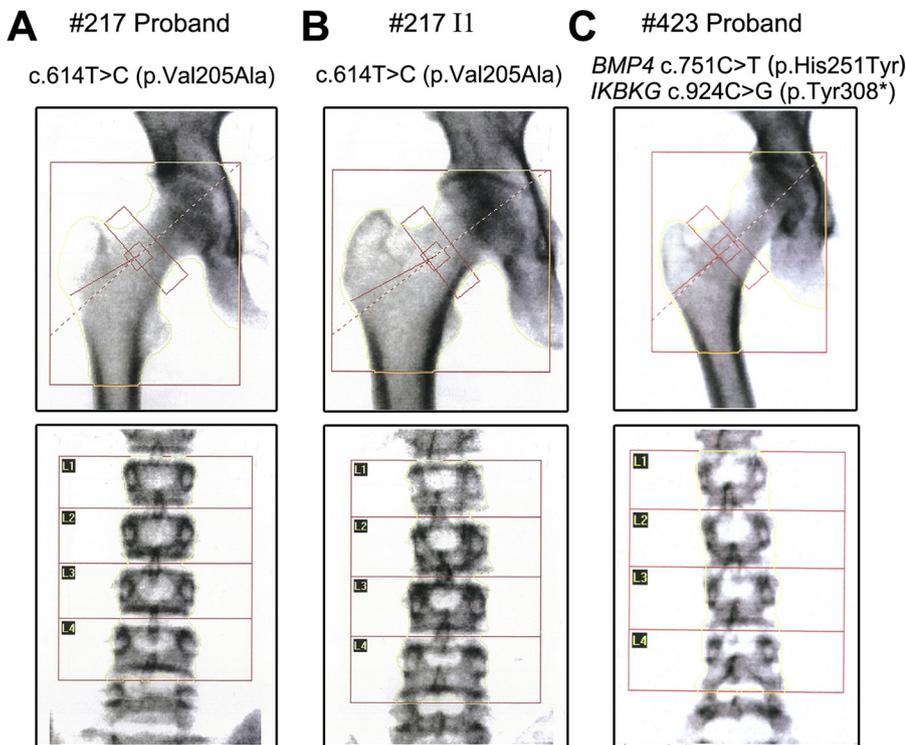
patients with a *BMP4* mutation had any bone diseases (Fig. 3).

Although the height and nutritional status of #217 proband (*BMP4 c.614 T > C*, p.Val205Ala) was within the normal range, the BMD of his lumber spine was 0.765 g/cm² (Table 3), which was significantly lower than the appropriate reference, and he was diagnosed with osteopenia. The BMD of the lumber spine in the #217 I1 (the proband's father) was 0.810 g/cm² (Table 3), which was significantly lower than the appropriate reference, and he was diagnosed with osteoporosis.

The BMD of the right femoral neck of #423 proband (*BMP4* c.751C > T: p.His251Tyr; *IKBKG* c.924C > G: p.Tyr308*) was 0.668 g/cm² (Table 3), which was significantly lower than the appropriate reference, and she was diagnosed with osteopenia. The patient's

Table 2
Schematic presentation of tooth anomalies in patients with a *BMP4* mutation.

Patient Number	Mutation	Right quadrants							Left quadrants						
		Max 7 / Mand 7	6 / 6	5 / 5	4 / 4	3 / 3	2 / 2	1 / 1	1 / 1	2 / 2	3 / 3	4 / 4	5 / 5	6 / 6	7 / 7
#217 II:1	BMP4 *p.Val205Ala*	■/■	■/■	□/□	□/□	■/□	■/□	□/■	■/■	■/□	□/■	□/■	■/□	□/■	■/■
#217 I:1	BMP4 *p.Val205Ala*	□/□	□/□	□/□	□/□	○/□	□/□	□/○	□/□	□/□	□/□	□/□	□/□	□/□	□/□
#290 II:1	BMP4 *p.Gly20Ser*	□/□	□/□	■/□	■/■	■/□	□/■	■/■	■/■	□/■	■/■	■/□	■/■	■/□	■/■
#551 II:1	BMP4 *p.Arg109Leu*	□/■	□/□	□/□	□/□	□/□	■/□	□/■	■/□	□/□	□/□	□/□	□/□	□/□	■/■
#69 II:1	BMP4 *p.His251Tyr*	□/□	□/□	■/■	□/□	◎/◇	◇/◇	◇/◇	◇/◇	◇/◎	◎/□	■/■	□/□	□/□	□/□
#69 I:1	BMP4 *p.Arg109Leu*	□/□	□/■	□/□	□/□	□/□	□/□	■/□	■/□	□/□	□/□	□/□	□/□	□/□	□/□
#423 II:1	BMP4 *p.His251Tyr*	■/□	□/■	■/□	■/■	□/□	□/△	■/△	■/△	□/□	■/■	■/□	□/■	■/□	■/□
#423 I:2	BMP4 *p.His251Tyr*	□/□	□/□	□/□	□/□	□/△	□/□	□/□	□/□	□/□	□/□	□/□	□/□	□/□	□/□

■ = the missing tooth; ○ = microdontia; △ = conical teeth; ◇ = narrow incisal edge; ◎ = slender cusp; □ = unaffected teeth

A #217 Proband
c.614T>C (p.Val205Ala)

B #217 I1
c.614T>C (p.Val205Ala)

C #423 Proband
BMP4 c.751C>T (p.His251Tyr)
IKBKG c.924C>G (p.Tyr308*)

Fig. 3. Dual-energy X-ray absorptiometry (DEXA) examination of lumbar and hip. (A) DEXA scan images of #217 proband carrying a *BMP4* mutation, c.614 T > C: p.Val205Ala, at the age of 15. (B) DEXA scan images of the father of #217 proband carrying the same *BMP4* mutation with #217 proband, at the age of 48. (C) DEXA scan images of #423 proband carrying a *BMP4* mutation, c.751C > T: p.His251Tyr, at the age of 20.

mother was not available for the DEXA examination.

3.3. Conservation analysis and preliminary functional studies

After identifying the 4 non-synonymous missense *BMP4* mutations in TA patients, we conducted a bioinformatics analysis to predict the effects of the *BMP4* mutations. We found three of the mutations, Arg109, Val205, and His251, were located in the TGF-beta propeptide region of the BMP4 protein (Fig. 4A). Amino acid sequence alignment of multiple species for the BMP4 protein showed that all affected residues were highly conserved during evolution (Fig. 4B). Next, *in silico* analyses were performed to predict the functional consequences caused by the missense mutations in *BMP4*. Among the 4 non-synonymous mutations, structure-homology based SIFT (Kumar, Henikoff, & Ng, 2009) and PolyPhen-2 (Adzhubei et al., 2010) predicted that 2 of the mutations, p.Val205Ala (V205A) and p.His251Tyr (H251Y), had a constant deleterious or probable damaging effect (Table 1). Therefore, they were selected for a preliminary functional study.

Next, we evaluated the possible functional changes of the BMP4 mutants, V205A and H251Y. Western blot analysis (Fig. S1) demonstrated that the expression levels of the BMP4 mutants, GFP-V205A and GFP-H251Y, were similar to that of WT GFP-BMP4 (Fig. S1A and B). Since Bmp4 has been shown to activate the Smad pathway to transduce signals for development and cell differentiation (Dexheimer et al., 2016; Salazar et al., 2016), the phosphorylation of R-Smads (Smad-1, Smad-5, Smad-9), members in the Smad signaling cascade, in cells

transfected with WT and mutant BMP4 plasmids was examined. Compared to WT-BMP4, the V205A and H251Y mutants had a significantly decreased level of phosphorylated Smad-1/5/9 in 293 T cells (Fig. S1C and D). Collectively, these data indicated that, while the V205A and H251Y mutants did not affect protein expression, they may impair BMP4's function since the mutants conferred a reduced activation of downstream Smad signaling pathway *in vitro*.

4. Discussion

Recent advances in sequencing technology have contributed an enormous amount of progress toward decoding the genetic aetio-pathogenesis of TA. To date, 15 genes have been reported to be responsible for non-syndromic TA, and a cluster of key genes has been identified as making a major contribution (91.9%) (Yu et al., 2019). However, only 1 mutation in the *BMP4* gene (p.Ala42Pro) has been reported to be associated with TA (Huang et al., 2013). Therefore, its role in the pathogenesis of human TA remains to be further investigated. In this study, we report 4 distinct *BMP4* mutations in 5 unrelated patients with TA. Among these *BMP4* mutations, 3 of them were novel mutations that were not previously reported to be associated with any genetic diseases. Interestingly, the *GREM2* gene, which encodes an antagonist protein to BMP4, was also an uncommon gene linked to isolated TA and tooth shape anomalies (Kantaputra et al., 2015). Therefore, our data corroborates with a previous study of a *BMP4* mutation contributing to tooth agenesis.

Table 3
Summary of DEXA results in patients with *BMP4* mutations.

Patient Number	Gender/ Age, y	Total BMC(g)	Total BMD(g/cm^2)	T-Score (adult) Z-Score (teenager)	WHO Classification
#217 proband	Male/ 15	Hip 29.56 Lumbar 45.61	Hip 0.780 Lumbar 0.765	Hip -1.6 Lumbar -1.1	osteopenia
#217 I 1	Male/ 48	Hip 30.54 Lumbar 57.12	Hip 0.750 Lumbar 0.810	Hip -1.9 Lumbar -2.6	osteoporosis
#423 proband	Female/ 20	Femoral Neck 3.01 Lumbar 47.79	Femoral Neck 0.668 Lumbar 0.948	Femoral Neck -1.6 Lumbar -0.9	osteopenia
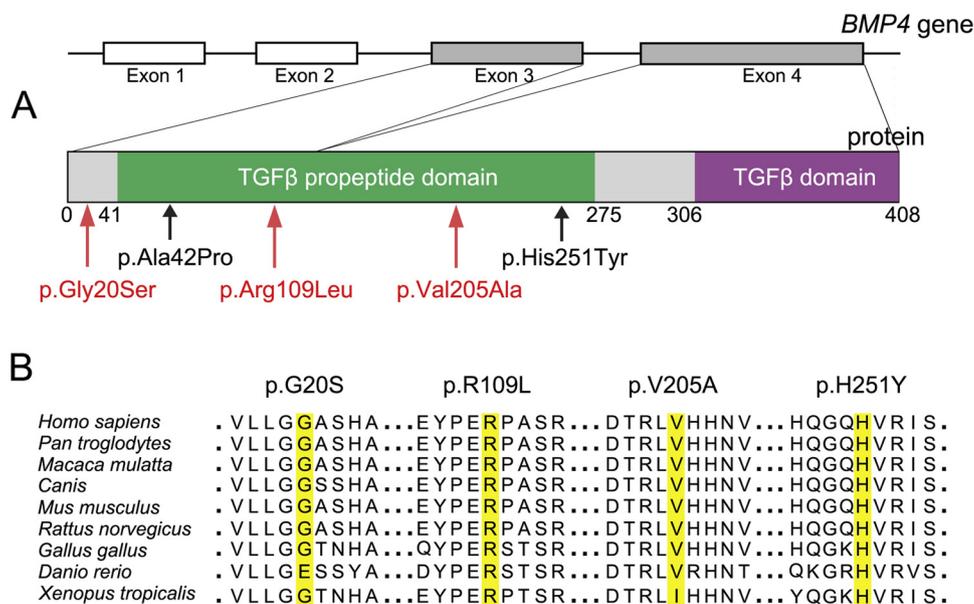
Fig. 4. Distribution and conservation analysis of tooth agenesis associated BMP4 mutations. (A) Schematic diagram of *BMP4* gene and protein structure showing all the BMP4 mutations identified in patients with TA. Novel mutations are marked in red, and reported mutation are in black. (B) Analysis of sequence conservation of involved BMP4 amino acids among different species.

One *BMP4* mutation (c.751C > T: p.His251Tyr) identified in our TA patients was previously linked to individuals with microphthalmia (Zhang et al., 2009) or hypospadias (Chen et al., 2007). However, we did not observe any of these symptoms in our patients. This result suggests that the *BMP4* mutations have inter-patient phenotypic heterogeneity. Heterozygous *BMP4* mutations have been shown to cause a board spectrum of clinical presentations, including orofacial clefts (Suazo et al., 2011), septal defects (Posch et al., 2008) and renal hypodysplasia (Weber et al., 2008). It is worthwhile to note that TA patients with a *BMP4* mutation in this study also presented remarkable intra-familial and inter-familial variabilities in dental manifestations, ranging from relatively mild tooth shape/size alterations or agenesis of third molars to severe oligodontia. Although the dental phenotypes found in patients carrying a *BMP4* mutation had variable clinical expressivity, they showed a complete penetrance with an autosomal dominant inheritance pattern.

Many animal studies have demonstrated that the Bmp superfamily plays an indispensable role in regulating skeletal development and homeostasis (Lowery & Rosen, 2018; Salazar et al., 2016; Wan & Cao, 2005) and that *Bmp4*'s expression in bones was consistently detectable from the embryonic stage to late adulthood in mice (Pregizer & Mortlock, 2015). Given that *BMP4* mutations resulted in an extensive phenotypic heterogeneity with varying symptoms and disease trajectories (Bakrania et al., 2008; Chen et al., 2007; Lubbe et al., 2011; Weber et al., 2008; X. Zhang et al., 2009) and that *Bmp4* is expressed in bones (Pregizer & Mortlock, 2015), we sought to explore the effects of *BMP4* on the skeleton. This study discovered that all of our patients with a *BMP4* mutation (n = 3) exhibited early onset osteopenia or osteoporosis, despite having normal growth without visible skeletal deformities. Therefore, our findings suggest that BMP4 may be essential for maintaining normal human bone density. Similar to tooth agenesis, familiar osteoporosis also has a strong genetic predisposition (Offiah, 2015). Loss-of-function mutations of proteins in the Wnt pathway, *WNT1* (Kampe, Makitie, & Makitie, 2015) and *LRP5* (Biha, Ghaber, Hacen, & Collet, 2016), have been shown to cause juvenile osteoporosis, while a heterozygous duplication of *BMP2* (Su et al., 2011) was found in a patient with brachydactyly, a congenital deformity of the digits. To our best knowledge, we demonstrate for the first time that bone mass density is reduced in individuals who carry a *BMP4* mutation, and thus we discover a new gene for early onset osteoporosis and osteopenia. However, the sample size of available patients was relatively small (n = 3) and one participant (#423 proband) carries digenic mutations in both *BMP4* and *IKBKG*, diluting the contributing effect

from a genetic aspect. Therefore, these findings need to be further confirmed.

Our preliminary functional studies indicated that two TA-associated BMP4 mutants were properly expressed *in vitro*, while the BMP4 mutants associated with renal hypodysplasia resulted in reduced protein expression or protein structural changes *in vitro* (Tabatabaeifar et al., 2009). However, we did find that the V205A and H251Y BMP4 mutants had defective Smad signaling activation, which is crucial for cell differentiation and organ development (Dexheimer et al., 2016; Lowery & Rosen, 2018; Salazar et al., 2016). Therefore, we speculate that a loss-of-function mutation in the *BMP4* gene may cause the dental and skeletal phenotypes observed in our TA patients. However, more comprehensive *in vitro* functional studies of all BMP4 mutants should be conducted using dental and bone cell lines, and the ultimate proof of pathogenicity of the identified missense mutations requires establishing rodent knock-in models by incorporating them with *BMP4* variants.

5. Conclusion

In summary, this study confirmed that *BMP4* is a reliable candidate gene for tooth agenesis. We also demonstrated that mutations in *BMP4* might be responsible for the early onset of osteoporosis or osteopenia in humans. These findings suggest that BMP4 may be involved in human tooth development and bone homeostasis.

Conflict of interest

The authors have no conflict of interest to declare.

Web resources

ExAC Browser, http://exac.broadinstitute.org/
OMIM, http://www.omim.org
PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/
SIFT, http://sift.jcvi.org/
Ensembl, http://www.ensembl.org/

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.archoralbio.2019.05.012.

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