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ORIGINAL ARTICLE

Patterns of molar agenesis associated with p.P20L and p.R77Q variants in *PAX9*

Narin Intarak[1] | **Thanakorn Theerapanon**[1] | **Thantrira Porntaveetus**[1] | **Vorasuk Shotelersuk**[2,3]

[1] Genomics and Precision Dentistry Research Unit, Department of Physiology, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand

[2] Center of Excellence for Medical Genomics, Medical Genomics Cluster, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

[3] Excellence Center for Genomics and Precision Medicine, King Chulalongkorn Memorial Hospital, the Thai Red Cross Society, Bangkok, Thailand

Correspondence
Thantrira Porntaveetus, Genomics and Precision Dentistry Research Unit, Department of Physiology, Faculty of Dentistry, Chulalongkorn University, Bangkok 10330, Thailand.
Email: thantrira.p@chula.ac.th

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Abstract

Nonsyndromic tooth agenesis is associated with variants in several genes. There are numerous genotype-phenotype publications involving many patients and kindreds. Here, we identified six Thai individuals in two families with nonsyndromic tooth agenesis, performed exome sequencing, and conducted functional experiments. Family 1 had four affected members carrying the heterozygous *PAX9* variant, c.59C>T (p.Pro20Leu). The p.Pro20Leu was previously reported in two families having four and three affected members. These seven cases and Proband-1 had agenesis of at least three third molars. Family 2 comprised two affected members with agenesis of all 12 molars. Both individuals were heterozygous for c.230G>A (p.Arg77Gln) in *PAX9*, which has not been reported previously. This variant is predicted to be damaging, evolutionarily conserved, and resides in the PAX9 linking peptide. The *BMP4* RNA levels in Proband-1′s leukocytes were not significantly different from those in the controls, whereas *BMP4* levels observed in Proband-2 were significantly increased. Moreover, the p.Arg77Gln variant demonstrated nuclear localization similar to the wild-type but resulted in significantly impaired transactivation of *BMP4*, a *PAX9* downstream gene. In conclusion, we demonstrate that the *PAX9* p.Pro20Leu is highly associated with absent third molars, while the novel *PAX9* p.Arg77Gln impairs *BMP4* transactivation and is associated with total molar agenesis.

KEYWORDS
hypodontia, microdontia, nonsyndromic, oligodontia, teeth

INTRODUCTION

Tooth agenesis is one of the most common congenital anomalies in humans with a prevalence ranging from 2.5 to 13.4% [1,2]. The congenital absence of 1–6 teeth is termed hypodontia, whereas the absence of more than six teeth is generally referred to as oligodontia. Anodontia is a rare condition with all teeth absent. Tooth agenesis can occur as an isolated condition (non-syndromic tooth agenesis) or manifest as a part of a syndrome, such as Kabuki syndrome, ectodermal dysplasia, skeletal dysplasia, and Axenfeld-Rieger syndrome [3–5].

Nonsyndromic tooth agenesis can be caused by pathogenic variants in several odontogenic genes, including *MSX1* [6], *AXIN2* [7], *PITX2* [8], *EDA* [9], *WNT10A* [10], and *PAX9* [11]. The paired box 9 (*PAX9*) gene is a member of the paired-box family of transcriptional factors that regulate organogenesis and neural crest cell development. It is located on chromosome 14 (14q13.3) and consists of five exons. The PAX9 protein contains the highly conserved paired DNA binding domain (N-terminal subdomain [NSD], C-terminal subdomain [CSD]), and octapeptide motif. The *PAX9* gene is expressed in the somites, pharyngeal pouches, and mesenchymal tissues during craniofacial, tooth, and limb development [12]. *Pax9* deficient mice demonstrated arrested tooth development at the bud stage corresponding with *Bmp4* downregulation in the dental mesenchyme, indicating that in human odontogenesis *PAX9* is necessary for *BMP4* expression [12].

The *PAX9* gene is one of the most common genetic causes of nonsyndromic tooth agenesis, second only to the *WNT10A* gene [12–14]. The reported *PAX9* variants comprise missense, frameshift, nonsense, and deletion variants and present as diverse clinical spectra dominated by molar tooth agenesis just as microdontia is frequently observed [15,16]. However, expanding the genotype-phenotype correlations of each *PAX9* variant requires more individuals to be studied. In this study, we determined the dental features of six individuals in two families with nonsyndromic tooth agenesis and identified *PAX9* variants by exome and Sanger sequencing. Functional studies were performed to substantiate its pathogenicity.

MATERIAL AND METHODS

Participants

Two unrelated patients with oligodontia and their family members were recruited from the Faculty of Medicine, Chulalongkorn University. Three age- and sex-matched healthy individuals were included in a control group for each patient. Informed consent was obtained from all participants. The study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn, Thailand (IRB No. 813/63) and was conducted in accordance with the Declaration of Helsinki (version 2002) and the additional requirements. Tooth number was reported using the FDI World Dental Federation numbering system.

Variant analysis

Genomic DNA was extracted from peripheral blood leukocytes [17,18]. Exome sequencing was performed using a TruSeqExome Enrichment Kit and Illumina Hiseq2000 at Macrogen Inc. with VariantStudio version 3.0.12 (Illumina). All variants were filtered with the databases comprising 1000 Genomes Project Consortium, Genome Aggregation Database (gnomAD), dbSNPs, T-REx [19] and in-house database of 2166 Thai exomes (including at least 1000 healthy individuals) [20]. The variants located in a coding region or canonical splice site were screened using the gene list in the Human Phenotype Ontology HP:0009804 that is related to tooth agenesis or absence of one or more teeth from the normal series by a failure to develop [21]. The variant was defined as novel if it was not present in the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php), gnomAD, and dbSNPs.

The identified variants were validated by Sanger sequencing using primer 1 (F: CCGATTGGACAGTGACGGTT and R: TGCTTGTAGGTCCGGATGTG) or primer 2 (F: AGCTGGGAGGAGTGTTCGT and R: GCTGGTGCTGCTTGTATGAG). Variant pathogenicity was predicted by InterVar [22], Mendelian Clinically Applicable Pathogenicity (M-CAP) [23] and Rare Exome Variant Ensemble Learner (REVEL) [24]. Alignment of the conserved regions among different species comprising *Homo sapiens* (NP_006185.1), *Mus musculus* (NP_035171.1), *Danio rerio* (NP_571373.1), *Drosophila melanogaster* (Pox meso, NP_001036687.1), and *Caenorhabditis elegans* (Pax-1, NP_505120.1) was performed by Clustal Omega (version 1.2.4).

Recombinant plasmids

Recombinant plasmids were purchased from GeneScript. The Myc-tagged pcDNA3.1 expression vector contained PAX9 wild-type or p.Arg77Gln. The pGL4.10 luciferase reporter vector was purchased from Promega (Catalog number: E6651). The 3.3-kb *BMP4* promoter fragment was introduced into the pGL4.10 vector by GeneScript. Nucleotide sequences of all constructed plasmids were verified.

Gene expression

Total RNA was extracted from peripheral blood leukocytes. RNA extraction was performed using a QIAamp RNA blood mini kit (Qiagen) according to the manufacturer's instructions. The isolated RNA was subjected to cDNA synthesis using an iScript cDNA Synthesis Kit (Bio-Rad) with oligo (dT) primers. The mRNA level expression was determined using CFX Real-Time PCR Detection Systems (Bio-Rad) with primers for *PAX9* (F: GAACGGGTTGGAGAAGGGAG, R: TGTCCAGCAACATAACCAGAAG), *BMP4* (F: CCAAGCGTAGCCCTAAGCT, R: ATGGCATGGTTGGTTGAGTTG), and *18S* rRNA (endogenous control). Each experiment was carried out in triplicate. Gene expression levels in the proband were compared with those in the control group. The data from RT-qPCR was calculated as relative fold change using the 2^(-delta delta CT) method [25].

The bar graphs were presented as mean relative fold change ± standard deviation (SD).

Immunofluorescence

Chinese hamster ovary K1 (CHO-K1) (ATCC) cells were transfected with Myc-tagged wild-type or p.Arg77Gln. After 48 h transfection, the transfected cells were fixed with 4% paraformaldehyde and permeabilized using 0.1% Triton X-100 (Thermo Scientific). Non-specific binding was blocked with 1% bovine serum albumin (BSA). The Myc-tagged recombinant protein was detected with mouse anti-Myc monoclonal IgG antibody (Biolegend) (1:500). The secondary antibody, Alexa 488-tagged goat anti-mouse IgG antibody (Abcam) (1:1000), was used in combination with nuclei staining DAPI (Roche) (1:2000) and Phalloidin (Invitrogen) (1:500). The cells were visualized using a ZEISS LSM 900 confocal laser scanning microscope (Carl Zeiss Microscopy).

Cell transfection

CHO-K1 cells (ATCC CCL-61) were seeded in 12-well plates or 8-well chamber slides and cultured in Ham's Nutrient Mixture F12 Media (HyClone) supplemented with 10% fetal bovine serum, 100 mg/mL penicillin, and 100 mg/mL streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. After 24 h incubation, the cells were transfected using X-tremeGENE 9 DNA transfection reagent (Roche) according to the manufacturer's instruction.

Luciferase reporter assay

CHO-K1 cells were transiently transfected with pcDNA3.1_PAX9, pcDNA3.1_Arg77Gln or pcDNA3.1 and the pGL4_BMP4 luciferase reporter vector. A Renilla expression plasmid, pRL-SV40 (Promega), was used as an internal control reporter plasmid for a transient transfection assay. At 48 h after transfection, the transfected cells were harvested for luciferase activity measurement using the Dual-Glo luciferase assay system (Promega) according to the manufacturer's protocol. The Firefly luciferase was normalized to Renilla luciferase. The *BMP4* promoter activity was presented as fold change relative to the empty vector control. The experiments were performed in triplicate and repeated three times.

Statistical analysis

Data from RT-PCR was presented as mean ± standard deviation (SD) and from luciferase reporter assays as mean ± standard error of mean (SEM). Normally distributed data were analyzed with the unpaired t-test using GraphPad Prism 8 Software Package (GraphPad). Data that did not follow a normal distribution were analyzed with the Mann-Whitney U test. Significance was defined A P-value < 0.05 was taken to indicate statistical significance.

RESULTS

Phenotypic and genotypic features

Proband-1, an 18-year-old male, presented with missing teeth. The clinical and radiographic examination revealed the absence of 14 permanent teeth (18, 28, 38, 48, 17, 27, 37, 47, 15, 25, 12, 11, 21, and 31), a peg-shaped 22, retained deciduous teeth, a thick and high attached upper labial frenum, and a high arched palate (Figure 1A, B). His physical and intellectual development were normal. The proband's grandmother, mother, and aunt also had more than 10 missing teeth and microdontia (Figure 1C–F). They all reported having missing teeth since they were young. However, their dental records were not retrievable. Exome and Sanger sequencing identified the heterozygous missense variant, c.59C>T (p.Pro20Leu), in exon 2 of the *PAX9* gene (SCV001759960) in the proband, grandmother, mother, and aunt, but not the father (Figure 1G, H). The variant was segregated with the tooth agenesis phenotype.

Proband-2 was a 23-year-old female presenting with missing teeth. Her oral examination revealed agenesis of all molar teeth (18, 28, 38, 48, 17, 27, 37, 47, 16, 26, 36, and 46) and peg-shaped 22 (Figure 2A, B). No other medical problems were noted. The proband-2′s father also reported lacking all molars since he could remember, but having had other teeth extracted (Figure 2 C, D). The novel heterozygous missense variant, c.230G>A (p.Arg77Gln), in exon 2 of *PAX9* (SCV001426687) was identified in the proband and father, but not the unaffected mother (Figure 2E–F).

The p.Pro20Leu and p.Arg77Gln variants were predicted to be damaging based on M-CAP (scores 0.9880 and 0.9635, respectively) and highly pathogenic by REVEL (scores 0.969 and 0.968, respectively). The amino acid position Pro20 resided in the N-terminal subdomain (NSD) and the p.Arg77 in the linking peptide of the PAX9 protein. Both positions were highly conserved among several species including human, mouse, zebrafish, fruit fly, and nematode roundworm (Figure 3). According to the American College of Medical Genetics and Genomics and the Association for Molecular Pathology 2015 standards and guidelines [26], the p.Pro20Leu was pathogenic, fulfilling criteria PS1, PS3, PM1, PM2, PP1, PP3, and PP5 (Table 1), and the p.Arg77Gln was likely pathogenic, fulfilling criteria PM1, PM2, PP3, and PP5 (Table 1). The c.230G>A (p.Arg77Gln) variant was not

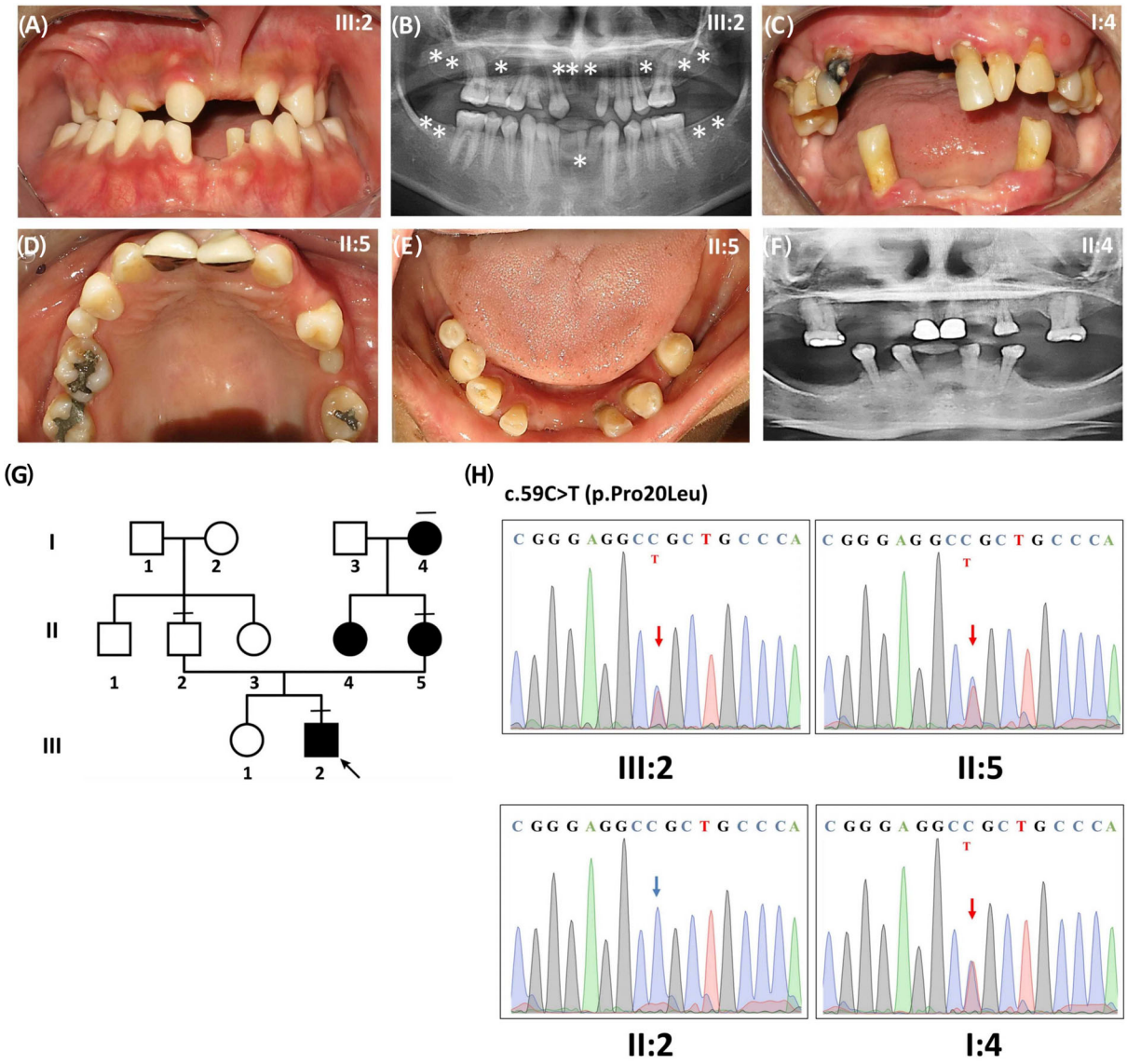


FIGURE 1 Pedigrees, genotype, and phenotype of Family 1. (A) Intraoral photograph of the proband (III:2) demonstrate the absence of multiple teeth, peg-shape maxillary left lateral incisor, retained deciduous teeth, and thick and wide labial frenum. (B) Panoramic radiograph reveals agenesis of nine maxillary teeth and five mandibular permanent teeth. Asterisks denotes the missing teeth. (C-F) Intraoral photographs and panoramic radiograph of the proband's grandmother (I:4), mother (II:5), and aunt (II:4) showing absence of multiple teeth and microdontia. (G) Family pedigree. Arrow indicates the proband. Black symbol indicates subjects with tooth agenesis. Dash indicates subjects recruited for genetic analysis. (H) Chromatogram demonstrating the heterozygous c.59C>T (p.Pro20Leu) variant in *PAX9* in III:2, II:5, and I:4, but not II:2

detected in the 1000 Genomes Project [27], gnomAD [28], in-house databases, and thus considered novel.

PAX9 expression in peripheral blood leukocytes

Analysis of the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) data demonstrated that the *BMP4* RNA levels in the Proband-1 were not significantly different from those in sex- and age-matched healthy controls while the *BMP4* levels in Proband-2 showed 3.38-fold increase compared with those in the controls (Figure 4A). We observed that the cycle threshold (Ct) values of *PAX9* in the probands and controls were above 35 and decided not to further analyze the *PAX9* expression data (Table S1).

Nuclear localization of the novel p.Arg77Gln variant

Immunofluorescence staining indicated that the p.Arg77Gln was predominantly localized in the nucleus resembling the localization of wild wild-type PAX9 (Figure 4C). This suggests that the p.Arg77Gln does not affect intracellular localization of PAX9.

European Journal of Oral Sciences

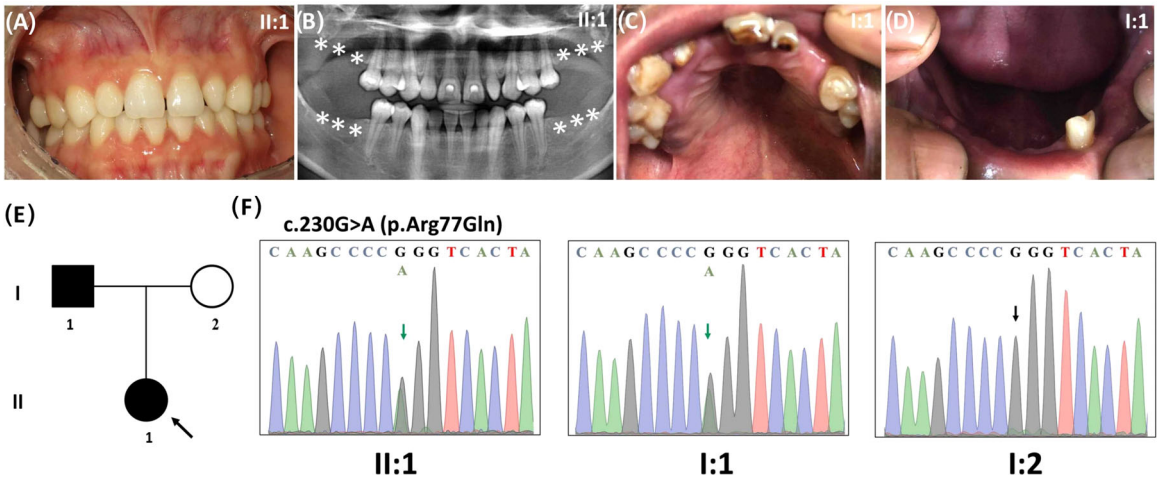
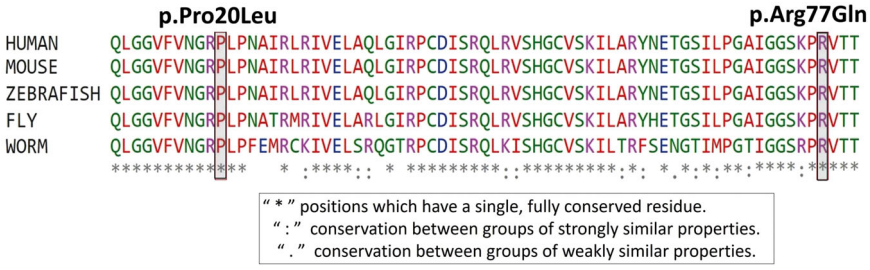


FIGURE 2 Pedigrees, genotype, and phenotype of Family 2 and amino acids conservation. (A, B) Intraoral photograph and panoramic radiograph of the proband showing the absence of all molars and peg-shape maxillary left lateral incisor. Asterisks denotes the missing teeth. (C, D) Intraoral radiograph of the proband's father (I:1) illustrating the absence of multiple teeth. (E) Family pedigree. Arrow indicates the proband. Black symbol indicates subjects with tooth agenesis. (F) Chromatogram demonstrates the heterozygous c.230G>A (p.Arg77Gln) variant in *PAX9* in II:1 and I:1, but not I:2

FIGURE 3 Amino acids conservation of PAX9. The p.Pro20 and p.Arg77 were highly conserved among multiple species



Transactivation activity of the p.Arg77Gln variant in CHO-K1 cells

BMP4 is a downstream gene of *PAX9*. To investigate the effect of the p.Arg77Gln on transcriptional activation, a dual luciferase reporter assay was employed using *BMP4*-luc as a reporter. The luciferase activity of p.Arg77Gln was significantly lower than that of wild-type PAX9 (Figure 4D), indicating that the p.Arg77Gln compromised the transcriptional activation ability of PAX9.

DISCUSSION

PAX9 variants have been found in 17% of nonsyndromic tooth agenesis cases [13,14]. Epidemiological studies usually exclude the third molars because they are frequently absent in the general population. However, we observed that the agenesis of all four third molars, which is rarely found in the general population, is common in patients with *PAX9* variants. To better represent the tooth agenesis phenotype of the *PAX9* patients, we included the third molars in our phenotypic evaluation. The heterozygous missense *PAX9* variant, c.59C>T (p.Pro20Leu), was observed in Family 1 with four affected members. The p.Pro20Leu was previously reported in two nonsyndromic tooth agenesis families, an Indian family with four affected members [29] and a Japanese family with three affected members [30] (Table 2). In total, there were eight cases identified with the p.Pro20Leu having definite records of third molars. All cases had oligodontia (≥ 6 missing teeth) and all, except for one who was missing three third molars, lacked four third molars (87.5%, 7/8). Microdontia or peg-shaped lateral incisors were observed in 6/10 cases (60%) (II:4 was excluded due to uncertain microdontia phenotype). These findings are consistent with previous studies, reviews, and meta-analyses demonstrating that the heterozygous *PAX9* variants are associated with certain tooth agenesis phenotypes, including (1) oligodontia, (2) nonsyndromic, (3) molar dominated, and (4) microdontia or peg-shaped lateral incisors [11,15,16,31,32]. Consistently, a review paper by Fournier et al. (2018) showed that the absence of upper and lower third molars was observed in 95% and 90% of the patients with *PAX9* variants, respectively. Regarding the number of missing teeth, the missing of upper

TABLE 1 Criteria for pathogenicity fulfilled by variants p.Pro20Leu and p.Arg77Gln according to Richards et al. [26]

Evidence of pathogenicity	Code	Description	Variant fulfilling
Strong	PS1	Same amino acid change as a previously established pathogenic variant regardless of nucleotide change	p.Pro20Leu
	PS3	Well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product	p.Pro20Leu
Moderate	PM1	Located in a mutational hot spot and/or critical and well-established functional domain without benign variation	p.Pro20Leu p.Arg77Gln
	PM2	Absent from controls in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium	p.Pro20Leu p.Arg77Gln
Supporting	PP1	Co-segregation with disease in multiple affected family members in a gene definitively known to cause the disease	p.Pro20Leu
	PP3	Multiple lines of computational evidence support a deleterious effect on the gene or gene product	p.Pro20Leu p.Arg77Gln
	PP5	Reputable source recently reports variant as pathogenic but the evidence is not available to the laboratory to perform an independent evaluation	p.Pro20Leu p.Arg77Gln

and lower third molars was accounted for 93.44% and 88.84% of missing teeth in the *PAX9* patients, respectively [16]. Previous functional study demonstrated that the p.Pro20Leu compromised the DNA binding and transactivation abilities of PAX9 [30], confirming its pathogenicity. Notably, according to the gnomAD [28], 1000 Genome Project [27], dbSNP [33], and HGMD [34] databases, the p.Pro20Leu has been reported only in Asians [29,30].

The novel heterozygous *PAX9* variant, c.230G>A (p.Arg77Gln) was identified in the Proband-2 and her affected father. Uniquely, both cases were only missing their molars (the father reported having all permanent molars absent and the other teeth were extracted). A peg-shaped lateral incisor was also observed in the Proband-2. The p.Arg77Gln changes arginine, an amino acid with a positively charged side chain, to a negatively charged glutamine. This could result in a conformational change in the paired DNA binding domain of PAX9 that interferes with its interaction with other odontogenic genes during tooth development. We identified that the p.Arg77Gln did not compromise the intracellular localization of PAX9, but significantly reduced the transactivation of BMP4, the downstream target of PAX9. Recently, the *PAX9* variant c.229C > G (p.Arg77Gly), adjacent to the c.230G > A, was reported in a patient who had 17 missing teeth (four third molars, four second molars, two first molars, three premolars, one canine, and two maxillary lateral incisors) [11]. The p.Arg77Gly reduced BMP4 transactivation and localized in the nucleus, similar to the p.Arg77Gln. Similarly, the other missense variants in the PAX9 paired domain also normally localized in the nucleus, but significantly reduced BMP4 expression [15,35]. In contrast, the truncating variants in the paired domain, although demonstrating reduced BMP4 transactivation, were localized in both the nucleus and cytoplasm [11]. Interestingly, the Proband-2 and her father were only missing their molars. To date, this phenotype was previously reported in only one case with the *PAX9* variant, c.59delC (p.Pro20Argfs65*) [36], which is in exon 2 and the conserved paired box sequence similar to the p.Arg77Gln. However, the correlation between these two variants and total molar teeth agenesis requires further study. Exon 2 comprises ~60% of the *PAX9* coding sequence. Most of the reported *PAX9* variants, including the two variants identified in this study, are in exon 2, indicating exon 2 as a mutation hotspot.

During tooth development, *PAX9* deletion results in downregulated mesenchymal *BMP4* expression [12]. However, how *PAX9* variants affect the expression of *PAX9* and *BMP4* in the peripheral blood leukocytes of the patients with tooth agenesis is unclear. A previous study found that a patient with nonsyndromic tooth agenesis and a heterozygous *PAX9* variant, c.1057G>A (p.Val111Met), had an upregulated *PAX9* mRNA level in their peripheral blood leukocytes, while *BMP4* was downregulated compared with that in control [37]. A study proposed that the higher expression of *PAX9* in the patient's leukocytes was due to the compensatory activation of the *PAX9* transcript [37]. The *PAX9* expression level in leukocytes was reported to be undetectable [38,39]. Accordingly, the cycle threshold values of *PAX9* in the probands and controls' leukocytes were above 35 (Table S1). The *PAX9* gene expression was then excluded from data analysis. Regarding *BMP4*, Proband-1 showed comparable expression levels with those in the controls while Proband-2 showed 3.38-fold increase compared with those in the controls. The expression of *BMP4* is directly and indirectly regulated by various genes and signaling molecules, including *PAX9, PITX2*, *MSX1*, and *MSX2* [40,41]. In addition, the consequence of *PAX9* variants on *BMP4* expression and tooth formation occurs at the early stage of odontogenesis or before the age

TABLE 2 Phenotypes of the patients with the p.Pro20Leu and p.Arg77Gln variants in PAX9

Patients	Tooth 18 48	17 47	16 46	15 45	14 44	13 43	12 42	11 41	21 31	22 32	23 33	24 34	25 35	26 36	27 37	28 38	N of missing teeth	Microdontia
c.59C>T (p.Pro20Leu)																		
Family 1 in this study																		
Proband 1 (III:2)	●	●		●			●	●	●	o			●		●	●		
	●	●							●						●	●	14	Yes
II:5	–			o		–					–		o	–		–		
	–	–	–	o				–	–			–		–	–	–	NA	Yes
II:4	–	–		–	–	–	–			–	–		–		–	–		
	–	–	–		–		–	–	–	–		–		–	–	–	NA	NA
I:4	–	–				–	–	–	o	o					–	–		
	–	–	–	–	–		–	–	–	–	–		–	–	–	–	NA	Yes
Thimmegowda et al. [29]																		
II:8	●						●			●						●		
	●															●	6	NA
III:9	●	●		●		●	●			●	●		●		●	●		
	●	●		●		●					●		●		●	●	18	NA
III:8	●						●			●						●		
	●															●	6	NA
III:7	●					●	●			●						●		
						●	●	●	●	●	●		●			●	13	NA
Murakami et al. [30]																		
ID7	●					●	o				o					●		
	●														●	●	6	Yes
ID9	●	●	●	●		o					o			●	●	●		
	●	●													●	●	11	Yes
ID10	●	●	●	●			o						●	●	●	●		
	●	●													●	●	12	Yes
c.230G>A (p.Arg77Gln)																		
Family 2 in this study																		
Proband 2 (II:1)	●	●	●							o				●	●	●		
	●	●	●											●	●	●	12	Yes

(Continues)

TABLE 2 (Continued)

Patients	Tooth 48	47	46	45	44	43	42	41	31	32	33	34	35	36	37	38	N of missing teeth	Microdontia
	18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28		
I:1	•	•	•					x			x			•	•	•		
	•	•	•	x	x	x	x	x	x	x		x	x	•	•	•	>12	NA

•, missing.
-, extraction or missing (dental records were not available).
o, microdontia/ peg-shaped.
x, extraction (dental record was confirmed).
NA, Not available.

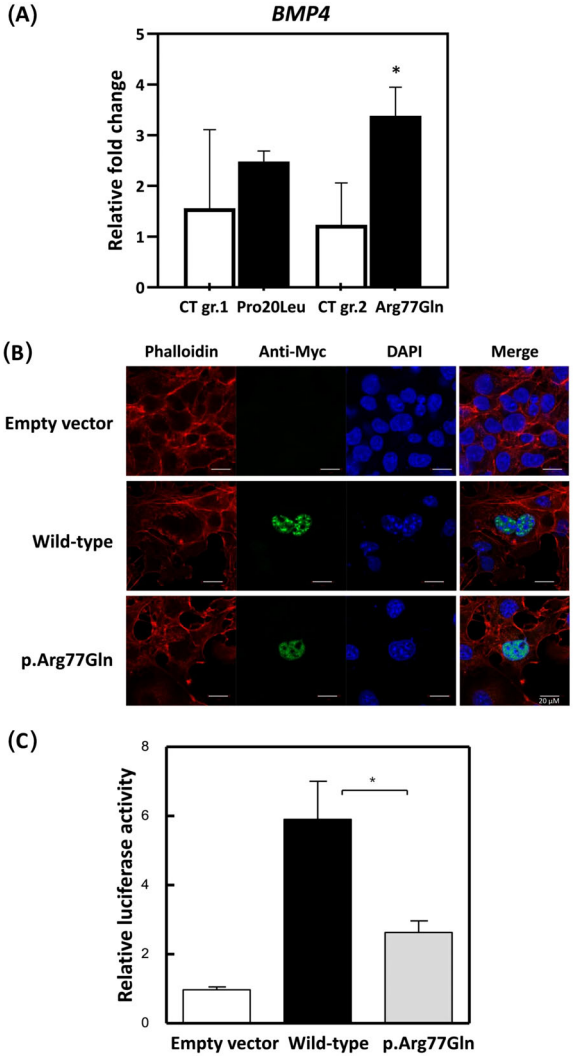


FIGURE 4 mRNA expression of *BMP4* in peripheral blood leukocytes and functional analyses of the p.Arg77Gln variant. (A) The bar graphs showed the *BMP4* expression levels in the patient with the p.Pro20Leu or p.Arg77Gln (black bars) compared with those in controls (white bars). Each control group comprise the RNA from three age- and sex-matched healthy individuals to each proband. The data is presented as mean relative fold change ± SD. (B) Immunofluorescence demonstrating the nuclear localization of the p.Arg77Gln localization similar to wild-type. Scale bar = 20 μM (C) Luciferase reporter assay on the *BMP4* promoter showed significantly reduced transcriptional activity of the p.Arg77Gln compared with control. *$P < 0.05$. gr., group

of 12−16 years (permanent dentition) [12,42]. The *BMP4* expression levels in leukocytes were significantly decreased in the patient with the *PAX9* variant p.Val111Met [37], not significantly different in the p.Pro20Leu, and significantly increased in the p.Arg77Gln, compared with those in the controls. These suggest that *BMP4* expression level in leukocytes might not directly correlate with the inherited *PAX9* variants. The mechanisms between different *PAX9* variants and the *BMP4* expression in leukocytes require further study.

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Homozygous hypomorphic *Pax9* mutant mice lack third molars and mandibular incisors. When the hypomorphic allele was combined with the null allele, molar development arrested at an earlier stage compared with that found in the two hypomorphic alleles [43]. Although the heterozygous *Pax9* mutant mice had normal tooth development, the heterozygous *PAX9* variants in humans led to nonsyndromic tooth agenesis. The truncating *PAX9* variants correlate with a more severe tooth agenesis phenotype compared with the missense variants [15,44,45]. It was proposed that the combined activity of the wild-type and mutant alleles less than the necessary level for normal tooth development is the cause of human nonsyndromic tooth agenesis [44]. The genotype-phenotype correlation of *PAX9* indicates that tooth agenesis severity is associated with type of variant or degree of *PAX9* loss-of-function and suggests that *PAX9* is a dosage-sensitive gene for tooth development. However, variable expressivity can be observed among patients with the same *PAX9* variants, suggesting that other unidentified factors, such as modifier genes, allelic variation, and genetic-environmental interactions might also contribute to the phenotypes [46].

In summary, we demonstrate that the PAX9 p.Pro20Leu variant is highly associated with oligodontia including at least three third molars, while the novel p.Arg77Gln variant, which impairs BMP4 transactivation, is associated with agenesis of all molars.

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AUTHOR CONTRIBUTIONS

Conceptualization: N. Intarak, T. Porntaveetus; **Investigation**: N. Intarak, T. Theerapanon; **Formal analysis**: N. Intarak, T. Theerapanon, T. Porntaveetus, V. Shotelersuk; **Writing – original draft preparation**: N. Intarak, T. Porntaveetus; **Funding acquisition**: T.Porntaveetus, V. Shotelersuk. All authors gave their final approval and agreed to be accountable for all aspects of the work.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ORCID

Narin Intarak https://orcid.org/0000-0001-7504-1403
Thanakorn Theerapanon https://orcid.org/0000-0001-6727-862X
Thantrira Porntaveetus https://orcid.org/0000-0003-0145-9801
Vorasuk Shotelersuk https://orcid.org/0000-0002-1856-0589

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SUPPORTING INFORMATION

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