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Detection of novel variant and functional study in a Chinese family with nonsyndromic oligodontia

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Abstract

Objectives: To investigate the pathogenic gene of a patient with nonsyndromic oligodontia, and analyze its possible pathogenic mechanism.

Subjects and methods: The variant was detected by whole exome sequencing (WES) and Sanger sequencing in a family with oligodontia. Bioinformatic and structural analyses were used to analyze variant. Functional studies including western blotting and immunofluorescent analyses and luciferase reporter assay were conducted to explore the functional effects.

Results: We identified a novel frameshift variant of *PAX9* (c.491-510delGCCCTATCACGGCGGCGGCC, p.P165Qfs*145) outside the DNA-binding domain causing an autosomal-dominant nonsyndromic oligodontia in a Chinese family. Bioinformatic and structural analyses revealed that the variant is pathogenic and conserved evolutionarily, and the changes might affect protein stability or folding. Functional studies demonstrate dramatically reduced ability in activating transcription activity of *BMP4* promoter and a marked decrease in protein production, as evaluated by western blotting and immunofluorescent analyses.

Conclusions: We found a novel frameshift variant of *PAX9* causing nonsyndromic oligodontia in a Chinese family. Our findings indicate that frameshift variants cause loss of function of PAX9 protein during the patterning of the dentition and the subsequent tooth agenesis, providing new molecular insights into the role of frameshift variant of *PAX9* and broaden the pathogenic spectrum of *PAX9* variants.

KEYWORDS
frameshift variant, functional studies, oligodontia, structural change, tooth agenesis, whole exome sequencing

1 | INTRODUCTION

Tooth agenesis is one of the most common oral and maxillofacial developmental anomalies in humans, which mostly occurs in the permanent dentition. There are obvious ethnic and regional differences, with an average prevalence rate of 6.53±3.33% (Nieminen, 2009; Rakhshan & Rakhshan, 2015). According to the number of missing teeth (excluding third molars), tooth agenesis can be divided into: hypodontia (no more than 6 teeth are missing), oligodontia (six or more teeth are missing), and anodontia (all teeth are missing). According to whether there are concomitant symptoms, tooth agenesis can be classified as isolated or nonsyndromic and syndromic dental agenesis. Studies have shown that although environmental factors (such as trauma, infection, and drugs) can affect the development of teeth,

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genetic factors are the most important pathogenic factors for congenitally missing teeth (Nieminen, 2009; Polder, Van't Hof, Van der Linden, & Kuijpers-Jagtman, 2004; Woolf, 1971). Syndromic dental agenesis is often due to a relatively clear genetic background and diagnostic basis, and the research methods are more mature, some have a specific pathogenic gene (Chhabra, Goswami, & Chhabra, 2014). However, nonsyndromic tooth agenesis is more complex with significant genetic and phenotypic heterogeneity and can be sporadic or familial. Various inheritance patterns of familial tooth agenesis include autosomal dominant inheritance, autosomal recessive inheritance, and X-linked inheritance have been reported (Das et al., 2002).

To date, there are about 20 genes associated with nonsyndromic tooth agenesis, mainly *PAX9*, *WNT10A*, *MSX1*, *WNT10B*, *LRP6*, *AXIN2*, and *EDA* (Dinckan et al., 2018; Yin & Bian, 2015; Yu, Wong, Han, & Cai, 2019). Among them, *PAX9* (GenBank accession number: NC_000014.9, paired box 9) is one of the most widely studied genes connected with odontogenesis. Stockton, Das, Goldenberg, D'Souza, and Patel (2000) first found that the *PAX9* variant could lead to the nonsyndromic tooth agenesis with autosomal dominant inheritance. In addition, polymorphisms in *PAX9* may increase the risk of hypodontia and oligodontia (Wang et al., 2013; Zhang, Qu, & Zhang, 2014).

PAX9, located on human chromosome 14 (14q13.3), is composed of five exons. A total of 1026 bases from exon 2 to exon 5 encode 341 amino acids of the protein, which consists of an N-terminal paired DNA-binding domain (PD), an octapeptide motif (OM), and a C-terminal transcriptional regulatory domain (Wang et al., 2009). As a transcription factor, the protein encoded by *PAX9* is co-expressed with *Msx1* (GenBank accession Number:NC_000004.12, msh homeobox 1) in dental mesenchyme during early tooth morphogenesis (Kapadia, Mues, & D'Souza, 2007). *PAX9* cannot only activate *Msx1* and *BMP4* (GenBank accession Number:NC_000014.9, bone morphogenetic protein 4) separately, but also interact with MSX1 to synergistically activate the *BMP4* gene promoter. An in vitro study demonstrated that *Pax9* homozygous null mice lacks derivatives of the third and fourth pharyngeal arches, has craniofacial and limb abnormalities, and fails to form teeth after the bud stage (Ogawa et al., 2006).

In this study, we intend to explore the pathogenic gene of a patient with nonsyndromic oligodontia, and analyze its possible pathogenic mechanism.

2 | MATERIALS AND METHODS

2.1 | Subjects

A female proband with congenitally missing teeth who visited the Department of Pediatric Dentistry in Peking University Hospital of Stomatology was recruited to this study. Based on an interview, general and oral examination, blood sample collection and radiographic examination, the proband and her affected family members were clinically diagnosed with nonsyndromic oligodontia. In addition, 50 healthy individuals were used as controls. The study protocol was approved by the Ethics Committee of Peking University School and Hospital of Stomatology (PKUSSIRB-202165093) and written informed consent from all participants was obtained.

2.2 | Whole exome sequencing (WES) and variant analysis

Genomic DNA was extracted using the TIANamp Genomic DNA Kit (Qiagen) as per instructions. After polymerase chain reaction (PCR), DNA products were sent for WES by the Novogene Bioinformatics Institute (Beijing, China). Then, we filtered the candidate genes using the following strategies with reference to GRCh37. First, the nonsynonymous single nucleotide variants (SNVs) and insertions/deletions (InDels) with a MAF <0.01 in 1000 genomic data, esp6500siv2_all, gnomAD data and in house Novo-Zhonghua exome database from Novogene were excluded in order to remove the diversity sites among individuals and obtain rare variants that may be pathogenic. Then, synonymous SNVs which are not relevant to the amino acid alternation predicted by dbscSNV were discarded and only SNVs occurring in exons or splice sites (splicing junction 10 bp) were retained for further analysis. Meanwhile, the small fragment (<10 bp) nonframeshift indel in the repeat region defined by RepeatMasker was ruled out. In short, variations that were predicted to potentially be deleterious according to scores of SIFT, Polyphen, MutationTaster, and CADD softwares or affect splicing were reserved. Second, we used the classification system of the American College of Medical Genetics and Genomics (ACMG) to better predict the harmfulness of variation by classifying them into five types: pathogenic, likely pathogenic, uncertain significance, likely benign, and benign (Richards et al., 2015). Finally, according to the above results and analysis, we conducted phenotype-based prioritization of candidate genes for human diseases by searching genes related to "tooth agenesis"/"congenitally missing teeth"/"oligodontia," and using DisGeNet database and Phenolyzer to sequence them so as to screen out the pathogenic gene variants of this family.

Next, to verify the variant and to analyze the familial cosegregation, the variant region's nucleotide sequence of *PAX9* was amplified using specific primers designed by primer design web site (www.ncbi.nlm.nih.gov/tools/primer-blast; primers listed in the Table S1). The PCR for *PAX9* was carried out under the following conditions: 5 min 95°C activation/premelt step, followed by 30 cycles of 30 s 94°C melt, 30 s 58°C anneal, and 60 s 72°C extension. After the target bands were qualified by 2.5% agarose gel electrophoresis, PCR products were sent to Tsingke Biological for purification and Sanger sequencing.

2.3 | Bioinformatics analysis

The conservation analysis of the variant among nine species was performed using the Clustal Omega (https://www.ebi.ac.uk/Tools/

msa/clustalo/). In addition, secondary structure of wild-type *PAX9* and variant *PAX9* was predicted by PsiPred 3.3 (http://bioinf.cs.ucl.ac.uk/psipred). Next, SWISS-MODEL (https://swissmodel.expasy.org) and MODELLER (https://salilab.org/modeller/) were used for homology modeling of the wild-type and variant PAX9 protein. Finally, we used the PyMOL Molecular Graphics System (DeLano Scientific) to visualize the change of variant PAX9 protein on the three-dimensional structure.

2.4 | Plasmid construction

The human *PAX9* cDNA (access number: NM_ 006194) was subcloned into pCDNA3.1 (+) vector with a C-terminal Myc-tag to construct the wild-type *PAX9* plasmid. Moreover, to further explore the possible pathogenesis, we also selected c.792-793insC (p.Val265fs), a frameshift variant also occurring outside the paired DNA-binding domain reported in previous literature, as a positive control (Frazier-Bowers et al., 2002). For the construction of frameshift variant pCDNA3.1(+)-P165Q-Myc and positive control plasmid pCDNA3.1(+) -Val265fs-Myc, we designed and synthesized specific primers to amplify templates by PCR, respectively , which can obtain the full-length sequence after variation, and then the fragments were subcloned into the expression vector pCDNA3.1 (+) with a C-terminal Myc-epitope. Next, the reporter plasmid pGL3-*PAX9* was constructed by subcloning 2.3 kb BMP4 promoter sequence into the NheI-HindII sites of pGL3-Basic vector (Promega). All the constructed plasmids were verified by DNA sequencing.

2.5 | Transfection of cultured cells

HEK 293T cells were cultured in Dulbecco's modified Eagle's medium(Gibco) supplemented with 10% fetal bovine serum (ABW) and 1% penicillin–streptomycin (Solarbio). All cultures were maintained in a humidified, 5% CO_2 atmosphere at 37°C. Lipofectamine 2000 (Invitrogen) was used for transient transfection of wild-type and variant plasmids according to the manufacturer's instructions. After 48 h of transfection, protein was harvested and analyzed by Western blotting or immunofluorescent analysis. Image J was used to convert the protein band to a gray value, and then GraphPad Prism8 was used to analyze the data with Student's *t* test. $P < 0.05$ was considered statistically significant.

2.6 | Western blotting analysis

Whole-cell lysates were prepared using RIPA Lysis Buffer and PMSF (Beyotime, Shanghai, China). Cytoplasmic and nuclear proteins were separated and extracted by NE-PER Nuclear and Cytoplasmic Extraction Reagents (78,833; Thermo). Twenty-five micrograms of whole-cell protein and 15 μg cytoplasmic or nuclear protein were measured by the BCA Protein Assay Kit (CW0014; Cwbio) and were separated from each group by 12.5% polyacrylamide gel for western blotting. Primary antibodies against Myc-tag (2276S; Cell Signaling Technology), PAX9 (658,202; Biolegend), β-tubulin (TA-10, ZSGB-Bio), and Histone H3 (AF0009; Beyotime) were used for overnight incubation at 4°C. After washing blots with TBST, secondary antibody (A0216; Beyotime) was used at room temperature for 1 h. Finally, ECL reagent (PE0010; Solarbio) visualized and imaged the protein bands. Image J was used to convert the protein band to a gray value, and then GraphPad Prism8 was used to analyze the data with Student's *t* test. $P < 0.05$ was considered statistically significant.

2.7 | Immunofluorescent analysis

Transfected cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with goat serum for 1 h. After overnight incubation with anti-Myc antibody ((2276S; Cell Signaling Technology) at 4°C, cells were washed with PBS and incubated with Fluorescein isothiocyanate (FITC)-labeled goat anti-mouse secondary antibody (ZF-0312; ZSGB-Bio) at room temperature for 1 h. Cells were sealed with mounting medium containing DAPI used to stain nuclei. The images were observed and photographed by TCS-SP8 STED 3X confocal microscope (Leica) and APO 63×/1.4 oil objective lens.

2.8 | *BMP4* promoter luciferase reporter assay

0.7 μg *PAX9* expression plasmid (including wild-type and variant) was co-transfected with 0.2 μg pGL3-*PAX9* reporter plasmid containing human *BMP4* promoter (p.2.3 BMP4-Luc) using Lipofectamine 2000 (Invitrogen). 0.1 μg of pRL-TK Renilla luciferase vector (Promega) was also co-transfected as an internal control for normalization. At 48 h after transfection, cells were harvested and measured with the Dual-Luciferase Reporter Assay kit (Promega) and Centro LB 960 Microplate Luminometer (Berthold Technologies, Bad Wildbad, Germany). The results were expressed in terms of relative luciferase activity that firefly luciferase activity was normalized based on renilla luciferase activity. Experiment was performed in triplicate and repeated three times. Use GraphPad Prism8 to analyze the data with Student's *t* test. $P < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Clinical findings

The proband was a 10-year-old girl who was diagnosed with nonsyndromic oligodontia on the basis of examination results. We analyzed 7 of 10 members (5 affected and 2 unaffected) of a three-generation family with oligodontia that segregated as an autosomal-dominant trait (Figure 1a). Neither the proband nor the family members had short stature or general health problems related to hair, nails, skin,

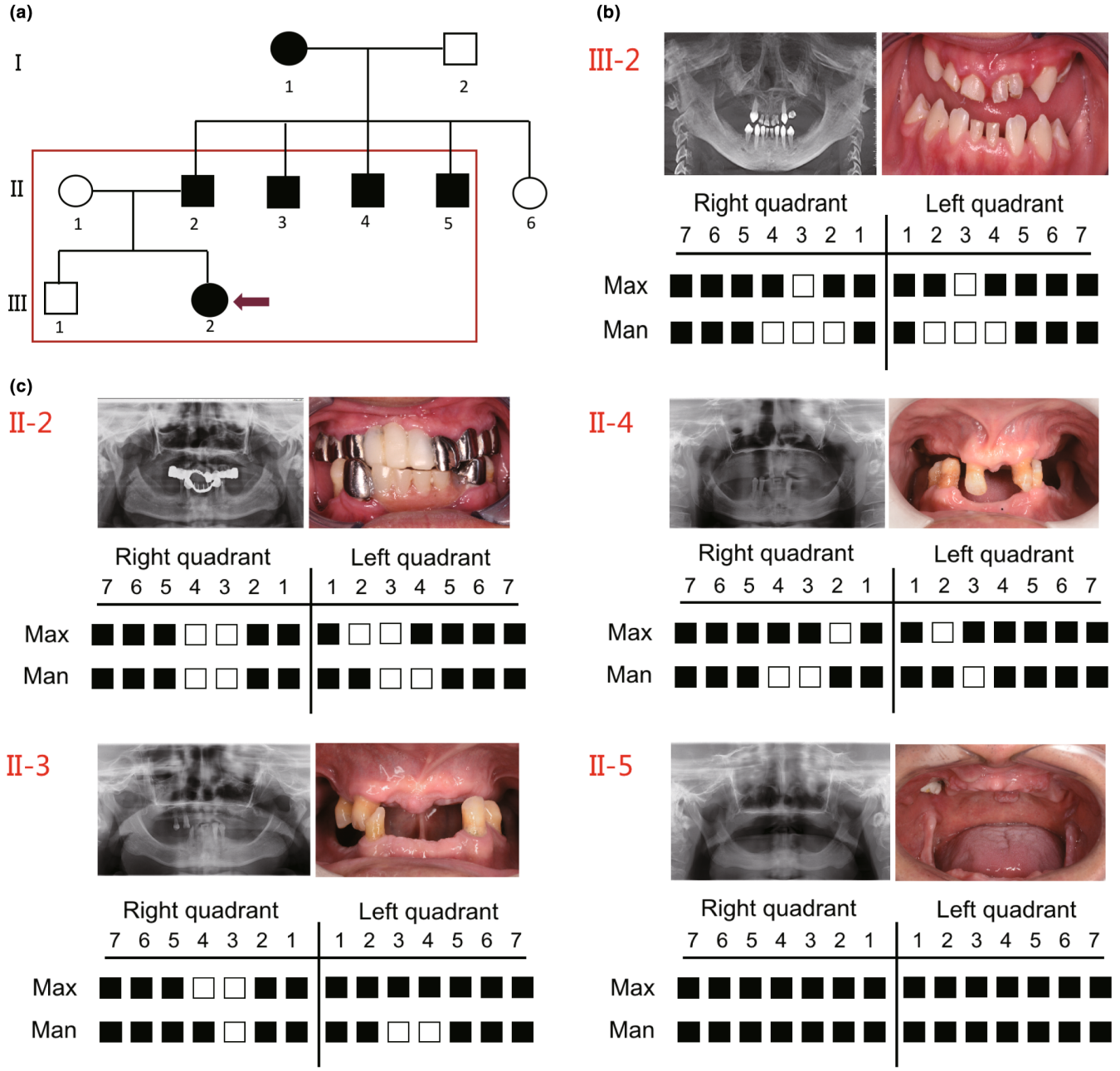


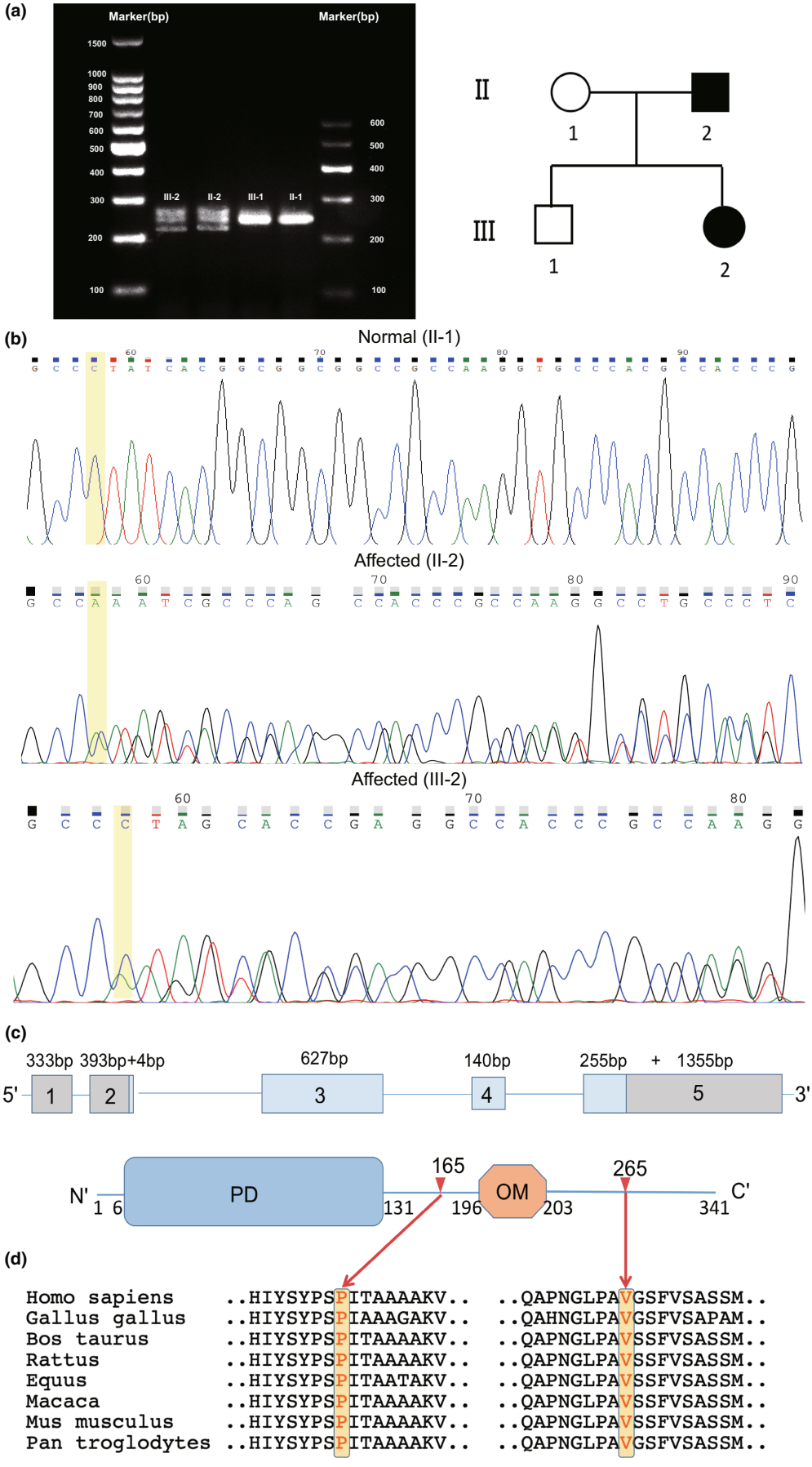
FIGURE 1 Clinical findings of the family. (a) the pedigree of the three-generation family with nonsyndromic oligodontia with the *PAX9* variant. Filled circles and squares indicate affected females and males. Arrows indicate the proband of the family. (b) cone beam CT, intraoral photos, and schematics of oligodontia in the proband (III-2). Max, maxillary; mand, mandibular (c) panoramic radiographs, intraoral photos, and schematics of oligodontia of affected family members with *PAX9* variants. Max, maxillary; mand, mandibular

and sweat glands (Table S2). The proband (III:2) had congenital loss of 20 permanent teeth, while the upper primary central incisors were fused teeth (Figure 1b). Four other affected members of her family (II:2, II:3, II:4, and II:5) lacked 20, 23, 28, and 23 permanent teeth, respectively (excluding third molars; Figure 1c and Table S3).

3.2 | Variant analysis

Whole exome sequencing revealed that a frameshift variant in *PAX9* with a 20-nucleotide deletion of exon 3 (c.491-510delGCCCT ATCACGGCGGCGGCC), which resulted in the subsequent amino

FIGURE 2 (a) Electrophoretic map of the mutant DNA fragment in patients (II-2/III-2:220/240 bp) and normal controls (II-1/III-1:240 bp). (b) sequencing chromatograms of available DNA in normal and affected members show a novel frameshift variant of *PAX9* (c.491-510delGCCCTATCACGGCGGCGGCC). The yellow marked part showed that the patients (II-2/III-2) have a heterozygous C-A base change in the sequence where the variant occurred, compared with the normal individual (II-1). (c) the schematic diagram shows cDNA of *PAX9* with boxes representing exons (1–5) as numbered. The diagram shows the amino acid sequence of PAX9 protein. (d)conservation analysis of affected amino acids in the PAX9 protein among 8 different vertebrate species



acid changes (p.P165Qfs*145) leading to the premature truncation of the protein at amino acid 308. Subsequently, horizontal gel electrophoresis (Figure 2a) and Sanger sequencing (Figure 2b and Table S4) confirmed this variant. The above-mentioned variant was neither detected in any of the unaffected relatives or healthy volunteers, nor has it been reported in 1000 Genomes project, dbSNP, ExAC or ClinVar databases, indicating that the *PAX9* variant identified in this study was a novel variant. According to ACMG classification, the variant was classified as pathogenic. In addition, the new *PAX9* variant, p.P165Qfs*145, is located between paired DNA binding-domain region and octapeptide motif region (Figure 2c). A conservation analysis revealed that the studied variants are highly conserved during evolution among different species (Figure 2d). Secondary structure analysis (Figure 3a) shows that the variant amino acid at position 165 changed from nonpolar and hydrophobic proline to polar and neutral glutamine, resulting in changes in the type, length, and structure of the subsequent amino acids. The results of tertiary structure analysis (Figure 3b) indicate that the folded structure of the variant protein changes outside the DNA-binding domain and produces a longer peptide chain than the wild-type protein.

3.3 | Effects of variant on PAX9 function

In order to explore the pathogenesis of tooth agenesis caused by mutated *PAX9*, we transfected the novel *PAX9* variant and the positive control *PAX9* variant that had been previously reported without functional study into HEK 293T cells, respectively, and studied biological function of the two *PAX9* variants in vitro. Western blotting revealed that wild-type and two frameshift variants could stably express the expected size of band (35–37 kDa) in vitro, but no band was seen in empty vector group (Figure 4a). However, the protein level was much lower in cell lysates of variant *PAX9* than that of the wild-type *PAX9*. To further verify whether the two frameshift variants could affect the protein stability, we transfected HEK 293T cells with Myc-tagged wild-type or variant *PAX9* and extracted the cytoplasmic and nuclear proteins, respectively. After protein quantification, we carried out western blotting experiment (Figure 4b). It was revealed that the expression of variant protein in cytoplasmic and nuclear both decreased compared with the wild-type protein. In addition, we detected subcellular localization by immunofluorescence to see whether the two frameshift mutations could affect the nuclear localization. Unlike the wild-type PAX9 localized in nuclei, the frameshift variants (p.P165Qfs*145 and p.Val265fs) were expressed in both cell nuclei and cytoplasm. However, the novel variant (p.P165Qfs*145) was mainly expressed in nucleus, while the positive control variant (p.Val265fs) was mainly in the cell cytoplasm (Figure 4c). Next, to determine whether the *PAX9* variants could affect the transcriptional activation of the *BMP4*, a downstream target of *PAX9*, we conducted a dual-luciferase reporter assay using *BMP4* promoter. The results showed (Figure 4d) that both activation of the reporter gene diminished or downregulated, indicating the mutated PAX9 proteins failed to activate the transcriptional activity of *BMP4*.

4 | DISCUSSION

So far, over 60 pathogenic variants of *PAX9* have been identified according to the ClinVar database and HGMD database, including about 30 variants related to tooth agenesis (Liang, Qin, Yue, He, & Bian, 2016; Wong et al., 2018; Zhang et al., 2019). PAX9 consists of a paired DNA-binding domain (PD) and an octapeptide motif (OM; Bonczek, Balcar, & Šerý, 2017). Among them, the paired domain is highly conserved and regulates the expression of target genes by binding to specific DNA sequences (Mensah, Ogawa, Kapadia, Cavender, & D'Souza, 2004). The octapeptide motif is also highly conserved and has transcriptional inhibitory activity (Eberhard, Jiménez, Heavey, & Busslinger, 2000). Given the role of PAX9 as a transcription factor, the variants mainly affect DNA-binding ability and transactivation activity of downstream target genes, leading to early retardation of tooth development (Chi & Epstein, 2002), resulting in nonsyndromic tooth agenesis characterized by molar loss. Moreover, *PAX9* is a dosage-sensitive gene in humans, with haploinsufficiency causing oligodontia. Previous study found that the severity of tooth agenesis was closely related to the residual DNA-binding capacity and promoter activation of the mutated PAX9 and that haploinsufficiency likely was the pathogenic mechanism for more severe clinical phenotypes (Wang et al., 2009; Wang, Groppe, et al., 2009).

In this report, we identify a novel frameshift variant in the *PAX9* gene in a Chinese family with nonsyndromic oligodontia. The variant localizes at the exon 3 of *PAX9* with 20 bases deletion (c. 491–510 delGCCCTATCACGGCGGCGGCC; p.S164fs/p.P165Qfs*145), which affects highly conserved amino acid residues and produces a premature termination of translation at amino acid 308. The prediction of secondary structure indicates changes in the properties of amino acids, leading to obvious changes of protein structure. The variant protein produces α-helix in advance and added 3 strands after the changed position compared with the wild-type protein, resulting in premature termination of translation by 33 amino acids in the variant protein. Three-dimensional structure prediction of PAX9 indicates that p.P165Qfs*145 variant has similar paired DNA-binding domain to the wild-type protein including two independent subdomains that structurally resemble a helix-turn-helix motif (Mensah et al., 2004; Xu et al., 1999). However, outside the DNA-binding domain, the folded structure of the variant protein changes and produces a longer peptide chain than the wild-type protein. Based on this, we speculate that this variant might affect protein stability or proper folding. Subsequent functional studies of the variant protein revealed that decreased protein expression whether in whole cell lysates or in cytoplasmic and nuclear proteins, dramatically reduced ability in activating transcription activity of *BMP4* promoter and defective nuclear localization that some of proteins were wrongly

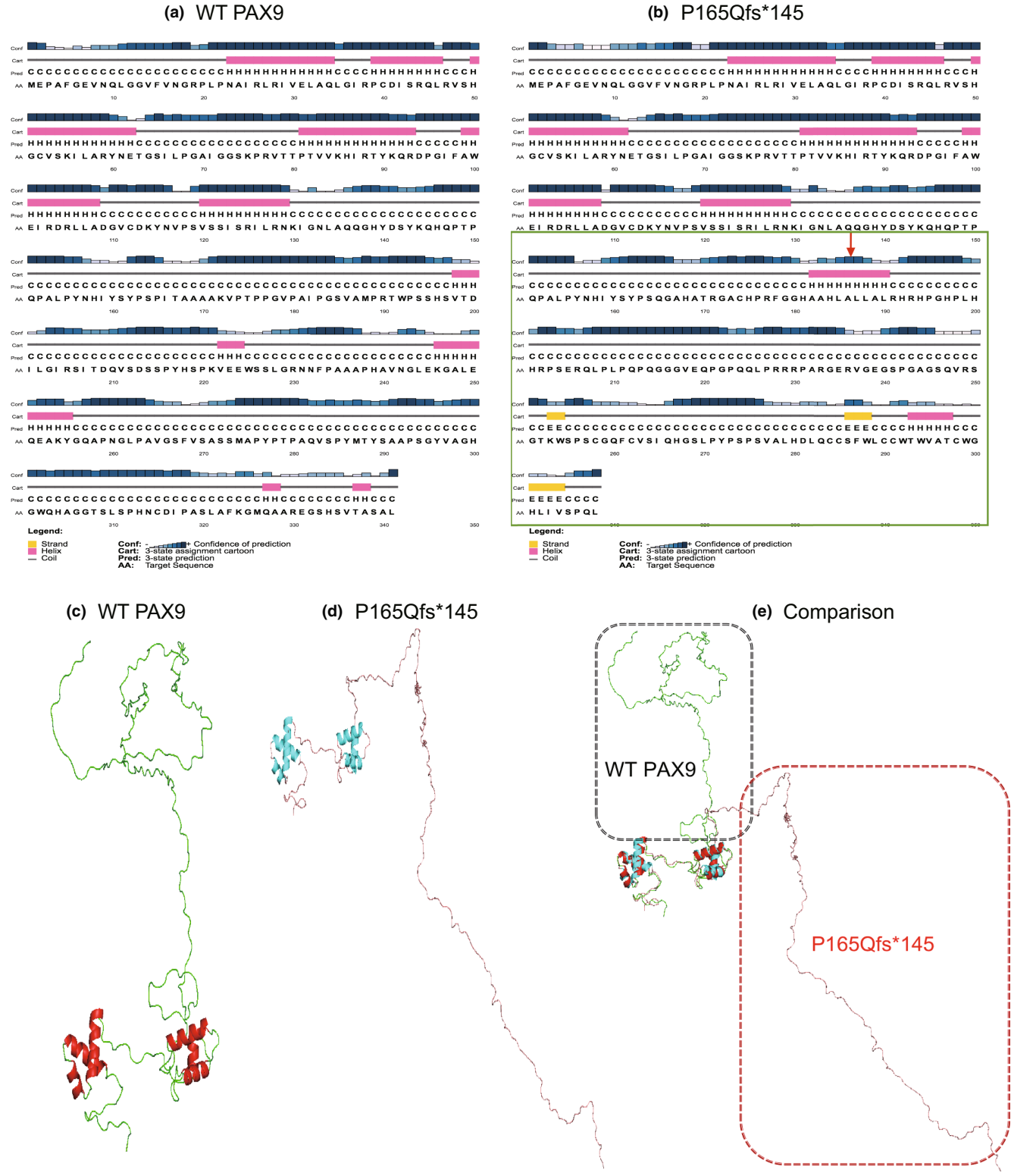


FIGURE 3 Structural analyses of wild-type and variant PAX9 p.P165Qfs*145. Secondary structural predictions of PAX9 indicate that the (a) wild-type and (b) variant protein of PAX9 were different. Tertiary structural modeling of wild-type (c) and variant PAX9 protein (d) and a comparison model after aligning the paired DNA-binding domains of wild-type and variant protein, difference of 3-dimensional structure outside the paired DNA-binding domain boxed with dotted lines, respectively (e)

located in the cytoplasm or perinuclear region. So, we come to the conclusion that p.P165Qfs*145 variant might affect protein stability and leads to the loss of function of PAX9 through eliminating the transactivation activity of PAX9 protein and changing nuclear location, resulting in haploinsufficiency during the patterning of the dentition and the subsequent tooth agenesis.

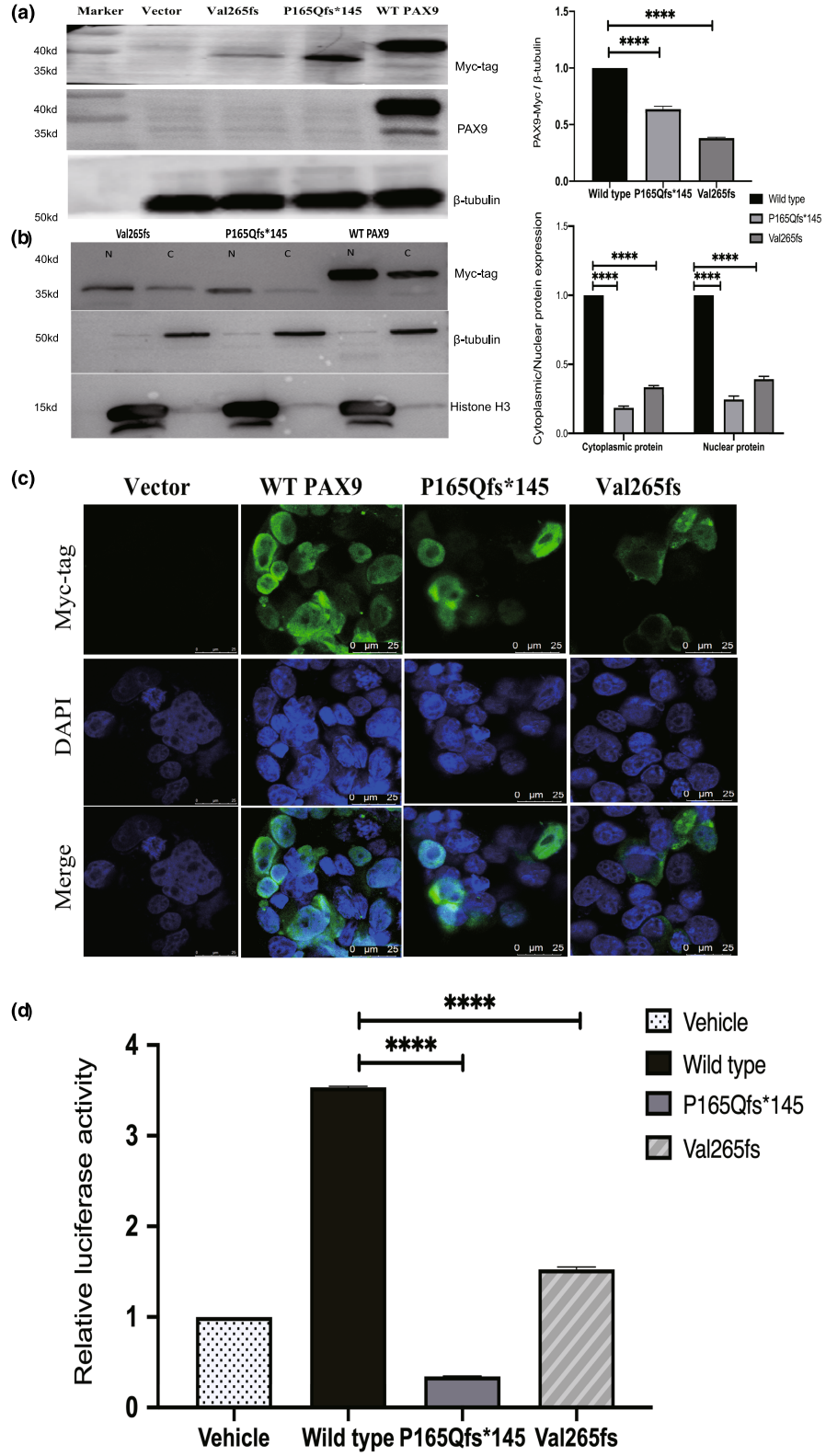


FIGURE 4 Functional studies of wild-type and variant PAX9 proteins. (a) the whole-cell expression of wild-type and variant PAX9 proteins detected by Western blotting using anti-PAX9, anti-Myc and anti-β-tubulin antibodies. Proteins with expected size of bands can be detected. ****$P<0.0001$. (b) the cytoplasmic and nuclear proteins of wild-type and variant PAX9 detected by Western blotting using anti-Myc, anti-β-tubulin, and anti-histone H3 antibodies. C, cytoplasmic protein; N, nuclear protein. ****$P<0.0001$. (c) subcellular localization of wild-type and variant PAX9 proteins evaluated by immunofluorescence. (d) the transcriptional activation abilities of wild-type and variant PAX9 proteins on the BMP4 promoter assessed by dual-luciferase reporter assay. ****$P<0.0001$

Efficient transactivation by PAX9 requires not only specific DNA-binding activity mediated by the paired domain but also transcriptional activation of amino acid residues within the C′-terminal domain (Mensah et al., 2004). The new PAX9 variant, p.P165Qfs*145, lying between PD region and OM region, does not affect the paired DNA binding-domain, but the amino acid residues of the octapeptide motif and the C′- terminal domain were changed after the variant site. The significant difference in transcriptional activation between the wild type and the p.P165Qfs*145 PAX9 suggests that the impact of the structural alterations caused by the 20 bases deletion on exon 3 extends from the octapeptide motif to the C'-terminal residue, thus affecting the correct activation of downstream target genes, such as *Msx1* and *Bmp4*.

TABLE 1 *PAX9* frameshift variants associated with tooth agenesis

Variant	Localization	Protein	Phenotype	References
c.59delC	Exon3	Pro20fs	Oligodontia	Mostowska, Zadurska, Rakowska, Lianeri, and Jagodziński (2013)
c.109-110insG	Exon3	Ile37fs	Oligodontia	Zhao, Chen, Bao, Wu, and Li (2005)
c.146delC	Exon3	Ser49Cysfs*36	Oligodontia	Wong et al. (2018)
c.176ins288bp	Exon3	Ala58fs	Oligodontia	Das et al. (2003)
c.185-189dup	Exon3	Gly64Argfs*23	Oligodontia	Wong et al. (2018)
c.218-219insG	Exon3	Gly73fs	Oligodontia	Stockton et al. (2000)
c.230-242del13bp	Exon3	Arg77Pro81delfs	Oligodontia	Bergendal, Klar, Stecksén-Blicks, Norderyd, and Dahl (2011)
c.256-262dup	Exon3	Arg88Profs*231	Oligodontia	Wong et al. (2018)
c.321-322insG	Exon3	Ala108fs	Oligodontia	Suda, Ogawa, Kojima, Saito, and Moriyama (2010)
c.353-354insTGCC	Exon3	Ser119fs	Oligodontia	Mostowska, Biedziak, Zadurska, Dunin-Wilczynska, and Lianeri (2013)
c.491-510del20bp	Exon3	Pro165Glnfs	Oligodontia	Present study
c.592delG	Exon3	Val198Serfs*	Oligodontia	Wong et al. (2018)
c.592-596dup	Exon3	Asp200dupfs	Oligodontia/ Hypodontia	Haddaji Mastouri et al. (2016)
c.619-621del ATCins24bp	Exon3	Ile207fs	Oligodontia	Mostowska, Biedziak, and Trzeciak (2006)
c.792_793insC	Exon5	Val265fs	Oligodontia	Frazier-Bowers et al. (2002)

Bold values indicate category items for indexing purposes.

BMP4 plays a vital role in the epithelial–mesenchymal interaction during dental morphogenesis (Vainio, Karavanova, Jowett, & Thesleff, 1993). *BMP4* variants and polymorphisms can lead to varying degrees of abnormal tooth development, ranging from relatively mild tooth shape/size changes to severe oligodontia (Yu et al., 2019). In addition, the subcellular localization of the variant PAX9 protein is impaired, which makes it unable to enter the nucleus and incorrectly localized in the cytoplasm or perinuclear region, resulting in a reduced dose of PAX9 and function loss as a transcription factor (Mensah et al., 2004). Our immunofluorescence results show that the mutated p.P165Qfs*145 PAX9 protein could be observed in both the nucleus and cytoplasm, in line with previous studies (Sun et al., 2021), clearly demonstrating that the function loss leading to the tooth agenesis could be partly due to the inadequate nuclear transport of the mutated protein.

Frameshift variants of *PAX9* are associated with varying degrees of function loss, with more severe tooth agenesis than missense variant but milder than nonsense variant (Wong et al., 2018). The novel frameshift variant we report here is most interesting, since it occurs outside the paired DNA-binding domain, the variant hotspots in the PAX9. Reviewing the literatures, variants occurred more often within the functional domain of PAX9, especially in the paired DNA-binding domain, and only 2 cases were located outside the functional domain (Table 1).

The first frameshift variant of *PAX9* occurred outside the functional domain was that insertion variant (c.792_793insC) located in exon5 (previously exon 4). The encoded protein terminates prematurely at amino acid 315 (p.Val265fs), causing the patients to suffer from molar oligodontia. The patterns of missing teeth of affected members showed considerable differences, but all showed missing molars. However, in addition to molar loss, some individuals also lack incisors and premolars, which is similar to the family in this study, indicating that the pattern of tooth agenesis is not limited to molars. Nevertheless, due to lack of experimental data, the possible pathogenesis could not be further clarified. In this study, the p.Val265fs variant is used as a positive control variant for functional research, in order to find out the effect of changes outside the functional domain of PAX9 protein on congenital tooth agenesis. Functional studies confirmed that although the proteins in whole-cell lysates, cell nuclei or cytoplasm of p.Val265fs can be stably expressed in vitro, the expression level is reduced compared with the wild type. In addition, the result of cellular immunofluorescence indicates that the variant protein is mislocalized in the cytoplasm and perinuclear region, which appears to be the root of the loss of function (Wang, Groppe, et al., 2009). More importantly, however, its downstream transcriptional activation of *BMP4* promoter is significantly reduced compared with that of the wild type. These results support that the transcriptional activation and nuclear translocation of p.Val265fs *PAX9* are impaired.

Therefore, we speculate that frameshift variants that occur outside the functional domain of the PAX9 protein can also lose the role of transcription factors by affecting the ability of transcriptional activation or nuclear translocation and protein structure. These findings indicate that frameshift variants cause loss of function of PAX9 protein during the patterning of the dentition and the subsequent tooth agenesis.

5 | CONCLUSION

We have identified a novel frameshift variant of *PAX9* contributing to nonsyndromic oligodontia in a Chinese family. Our research provides new molecular insights into the role of frameshift variants of *PAX9* leading tooth agenesis, and broadens the pathogenic spectrum of *PAX9* variants.

AUTHOR CONTRIBUTIONS

Ruiqing Sun: Conceptualization; data curation; formal analysis; writing – original draft. **Shuangying Li:** Data curation; resources; writing – review and editing. **Bin Xia:** Conceptualization; data curation; writing – review and editing. **Junxia Zhu:** Conceptualization; data curation; investigation; project administration; resources; supervision; validation; writing – review and editing.

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CONFLICT OF INTEREST

All of the authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in [repository name e.g "figshare"] at http://doi.org/[doi], reference number [reference number].

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/odi.14259.

PATIENT CONSENT STATEMENT

The study protocol was approved by the Ethics Committee of Peking University School and Hospital of Stomatology (PKUSSIRB-202165093) and written informed consent from all participants was obtained.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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