Received: 28 May 2023 | Revised: 3 September 2023 | Accepted: 28 November 2023

DOI: 10.1002/mgg3.2334

Molecular Genetics & Genomic Medicine Open Access WILEY

ORIGINAL ARTICLE

Characterization of novel *MSX1* variants causally associated with non-syndromic oligodontia in Chinese families

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Funding information
Natural Science Foundation of Hebei Province, China, Grant/Award Number: H2022206246; Special Project of Health Innovation of Hebei Provincial Department of Science and Technology of China, Grant/Award Number: 21377716D; Hebei Provincial Government Funded Clinical Medicine Talents Training and Basic Research Projects, Grant/Award Number: 361029

Abstract

Background: *MSX1* (OMIM #142983) is crucial to normal dental development, and variants in *MSX1* are associated with dental anomalies. The objective of this study was to characterize the pathogenicity of novel *MSX1* variants in Chinese families with non-syndromic oligodontia (NSO).

Methods: Genomic DNA was extracted from individuals representing 35 families with non-syndromic oligodontia and was analyzed by Sanger sequencing and whole-exome sequencing. Pathogenic variants were screened via analyses involving PolyPhen-2, Sorting-Intolerant from Tolerant, and MutationTaster, and conservative analysis of variants. Patterns of *MSX1*-related NSO were analyzed. MSX1 structural changes suggested functional consequences in vitro.

Results: Three previously unreported *MSX1* heterozygous variants were identified: one insertion variant (c.576_577insTAG; p.Gln193*) and two missense variants (c. 871T>C; p.Tyr291His and c. 644A>C; p.Gln215Pro). Immunofluorescence analysis revealed abnormal subcellular localization of the p.Gln193* MSX1 variant. In addition, we found that these *MSX1* variants likely lead to the loss of second premolars.

Conclusion: Three novel *MSX1* variants were identified in Chinese Han families with NSO, expanding the *MSX1* variant spectrum and presenting a genetic origin for the pathogenesis detected in patients and their families.

KEYWORDS
genotype–phenotype, *MSX1*, non-syndromic oligodontia, tooth agenesis, whole-exome sequencing

1 | INTRODUCTION

Tooth agenesis (TA) is a frequent developmental dental abnormality. In permanent teeth, TA incidence is 6%–7% in the general population (Rakhshan, 2015). Depending on whether it is accompanied by developmental abnormalities in other organs, TA can be divided into syndromic TA (STA) and non-syndromic TA (NSTA) (Zeng et al., 2019). Non-syndromic TA is further categorized according to the missing teeth, as follows: hypodontia (absence of 1–5 permanent teeth, which excludes the third molars), oligodontia (absence of ≥6 permanent teeth, which excludes the third molars), and anodontia (complete lack of teeth) (Wang et al., 2016). Most cases of TA stem from genetic variants, and variants in the *PAX9* (OMIM #167416), *WNT10B* (OMIM #601906), *MSX1* (OMIM #142983), *WNT10A* (OMIM #606268), *AXIN2* (OMIM #604025), *EDAR* (OMIM #604095), *EDA* (OMIM #300451), *EDARADD* (OMIM #606603), and *LRP6* (OMIM #603507) genes were linked to NSTA (Khan et al., 2022; Mumtaz et al., 2020; Song et al., 2020; Zeng et al., 2019; Zhang et al., 2021). Of these, *MSX1* was the first gene identified to cause NSTA.

The transcription factor *MSX1* (formerly known as HOX7) has a key regulatory function in tooth development and organogenesis. *MSX1* is commonly expressed in numerous organs, chiefly during bud- and cap-stage tooth development where epithelial–mesenchymal interactions ensue in odontogenesis (Thesleff, 2006). Msx1$^{-/-}$ mice showed complete secondary cleft palate, total failure of tooth development, and terminated molar development during the embryonic stage (Zheng et al., 2021). *MSX1* is located at 4p16.2, contains two exons (Tatematsu et al., 2015), and encodes the MSX1 protein, which is composed of 303 amino acids and contains a highly conserved DNA-binding homologous domain (HD).

In this study, representatives of 35 families with non-syndromic oligodontia (NSO) were screened by whole-exome sequencing (WES), and three novel *MSX1* variants were found. The functional consequences of the novel truncated variant were explored, and we analyzed the phenotype of patients with *MSX1*-related variants. These data demonstrate that *MSX1* variants are a theoretical functional basis for NSO, and propose potential mechanisms underlying the genotype–phenotype correlation of NSO caused by *MSX1* variants.

2 | MATERIALS AND METHODS

2.1 | Pedigree construction and clinical diagnosis

A 35-person cohort of non-consanguineous probands with NSO referred to the Hebei Medical University Hospital of Stomatology Department of Prosthodontics was recruited from 2017 to 2022. Prosthodontics specialists examined all participants to establish the status of their dentition, including taking panoramic radiograph images (Figure 1). The Ethics Committee of the School and Hospital of Stomatology, Hebei Medical University, approved this study (NO: [2016] 004) and written informed consent was obtained from all participants.

2.2 | Whole-exome sequencing and Sanger sequencing

We obtained peripheral venous blood samples (2 mL) from the probands and their family members. We extracted genomic DNA using a blood genomic DNA extraction kit (Beijing Tiangen Biochemical Technology) according to the instructions of the manufacturer and stored it at −20°C until use.

The probands' genomic DNA underwent WES (iGeneTech, Beijing, China). In brief, the WES process involved establishing a DNA library, quality inspection and quantification, and sequencing of the target region exons with the NovaSeq 6000 platform (Illumina Inc., CA, USA). We mapped sequence reads to the human reference genome hg19 (GRCh37) with the Burrows–Wheeler Aligner (v.0.7.17). ANNOVAR was used to identify small indels and single-nucleotide variants using SAMtools and the Genome Analysis Toolkit (GATK) pre-annotation.

We ascertained candidate variants based on the following criteria: (1) gene known to be pathogenic; (2) minor allele frequency (MAF) < 0.01 in the 1000 Genomic Project or Exome Aggregation Consortium (ExAC) data; and (3) predicted as pathogenic by PolyPhen-2, Sorting Intolerant from Tolerant (SIFT), or MutationTaster. The *MSX1* gene bidirectional primers contained the predicted pathogenic loci. The reference *MSX1* sequence is NM 002448.3.

The primer sequences we used were as follows:
MSX1-exon2-F:5′GGCGGCACTCAATATCTGG3′;
MSX1-exon2-R:5′CTCCAGCTCTGCCTCTTGTAG3′;
MSX1-exon2-F:5′CGCAAACACAAGACGAACCG3′;
MSX1-exon2-R:5′CACATGGGCCGTGTAGAGT3′;
MSX1-exon2-F:5′CAAACACAAGACGAACCGTAAG3′;
MSX1-exon2-R:5′CTATGTCAGGTGGTACATGCTG3′.

2.3 | Structural modeling and conservation of the MSX1 variants

In the analysis of conservation, Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustano) was applied to compare the MSX1 amino acid sequences of human (NP_002439.2),

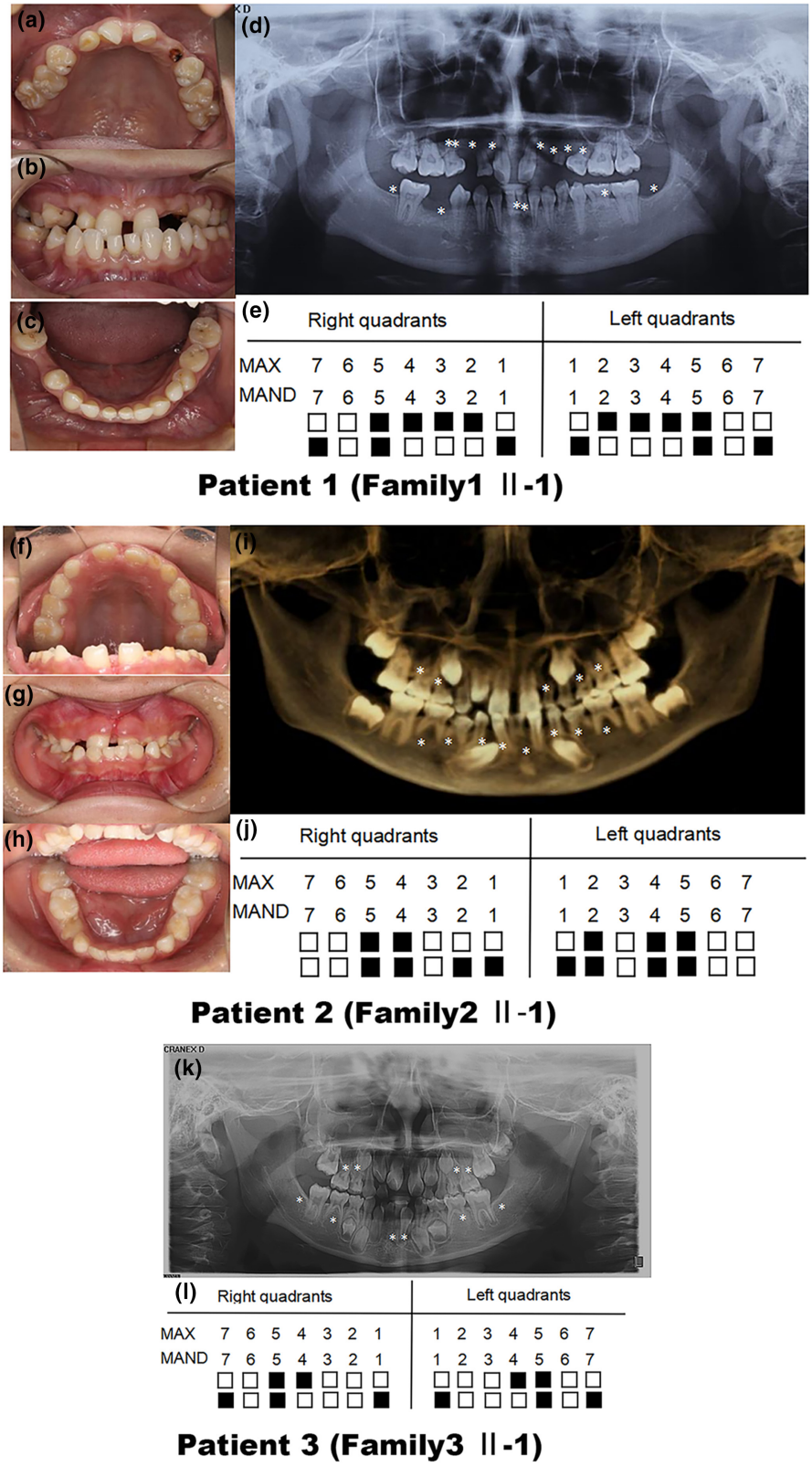

FIGURE 1 Dental characteristics of the three probands with non-syndromic oligodontia (NSO). (a–e) Intraoral images, panoramic radiographs, and legend of intraoral missing teeth in oligodontia in Family 1 proband; (f–j) Intraoral images, panoramic radiographs, and legend of intraoral missing teeth in oligodontia in Family 2 proband; (k,l) Panoramic radiographs, and legend of intraoral missing teeth in oligodontia in Family 3 proband. Asterisks and black squares indicate missing teeth; Max, maxillary; Mand, mandibular.

chimpanzee (NP_001182191.1), dog (XP_038516490.1), cow (NP_777223.1), mouse (NP_034965.2), cat NP_112321.2), and chicken (NP_990819.1).

Secondary structures of MSX1 were forecast with PsiPred 4.0 (http://bioinf.cs.ucl.ac.uk/psipred). The protein structure of MSX1 was acquired from the Protein Data Bank (http://www.rcsb.org/), and three-dimensional homologous structures were predicted by SWISS-MODEL (https://swiss-model.expasy.org). Three-dimensional (3D) structure visualization and structural change analysis

were conducted using PyMOL v2.1 (Molecular Graphics System, DeLano Scientific, CA, USA).

2.4 | Culture of cells, transient transfection, and immunofluorescence

We cultured human dental pulp stem cells (hDPSCs; Beijing Tason Biotech, Beijing, China) in Dulbecco's modified Eagle's medium (Gibco, USA) containing 10% fetal bovine serum (Gibco) at 37°C in humidified air.

The wild-type *MSX1* vector pcDNA3.1-3xFlag-MSX1 was donated by Professor Han (Peking University School and Hospital of Stomatology, Beijing, China). The variant vector pcDNA3.1-3xFlag-Gln193* was produced by Fenghui Biotech (Hunan, China).

The transient transfections were conducted using Lipofectamine 3000 (Thermo Fisher Scientific, MA, USA). At 48-h post-transfection, samples were incubated with anti-FLAG mouse-derived primary antibody (Sigma, USA), then were incubated with labeled fluorescent secondary antibody (Abways, China) in the dark, stained with 4′,6-diamidino-2-phenylindole (DAPI), then mounted. Images were captured with an Olympus FV1000 laser confocal microscope (Olympus, Japan).

2.5 | Genotype–phenotype analysis

PubMed studies covering *MSX1* variants that were published up to December 31, 2022, were identified for genotype–phenotype analysis. We excluded studies that did not report phenotype information in detail. We obtained the phenotype data of 44 *MSX1* variants in NSTA from the articles and from the three patients in this study for genotype–phenotype analysis. The number and rate of missing teeth were calculated.

3 | RESULTS

3.1 | Clinical findings for patients bearing new *MSX1* variants

We studied 35 patients with NSO from unrelated families. All patients were examined carefully and had a clinical diagnosis of NSO. The diagnoses were confirmed via intraoral examinations and panoramic radiographs.

In Family 1, the proband was a 17-year-old female. Clinical examination and panoramic radiography demonstrated that, as well as nine retained deciduous teeth, the proband lacked 14 permanent teeth (12, 13, 14, 15, 22, 23, 24, 25, 31, 35, 37, 41, 45, and 47; except for the third molar) (Figure 1a–e). The proband's parents were not found to congenitally lack permanent teeth.

In Family 2, the proband was a 9-year-old girl with mixed dentition who exhibited malocclusion. Clinical examination and panoramic radiograph demonstrated oligodontia of 13 permanent teeth, which included five incisors and eight premolars (Figure 1f–j). The shape and size of the remaining teeth were normal, and there was no history of tooth extraction. The proband's father was found to congenitally lack permanent teeth.

In Family 3, the proband was a 9-year-old boy with congenital agenesis of two anterior teeth, two molars, and six premolars. While members of Family 3 were unwilling to provide intraoral photos, the proband's father was found to congenitally lack permanent teeth (Figure 1k,l).

3.2 | Identification of novel variants

Whole-exome sequencing and Sanger sequencing identified a new insertion variant (c.576_577insTAG; p.Gln193*) and two novel missense variants (c. 871T>C; p.Tyr291His, and c. 644A>C; p.Gln215Pro) of *MSX1* (Figure 2). These variants were not found in the 1000G, dbSNP, ExAC, or gnomAD databases, which suggested that all the *MSX1* variants we identified are rare variants. According to ACMG guidelines (2015), p.Gln193* and p.Gln215Pro are predicted to be pathogenic/likely pathogenic, and p.Tyr291His is predicted to a variant of uncertain significance (Table 1).

The Family 1 proband bore a heterozygous insertion variant involving three amino acids, c.576_577insTAG(p.Gln193*), which caused a premature stop codon at amino acid 193 (Figure 2a). Through oral interviews, we learned that the proband's parents were healthy individuals, but their DNA samples were not obtained. The MSX1 protein amino acid sequence alignment shows that Gln193 is highly conserved in numerous species (Figure 2d).

FIGURE 2 Identification and conservation analysis of three novel variants of the *MSX1* gene in patients with non-syndromic oligodontia (NSO). (a) The pedigree of NSO Family 1. DNA sequencing chromatograms showed that compared with the wild-type control, a novel insertion variant, c.576_577insTAG (p.Gln193*), was identified in proband 1. (b) Pedigree of the affected Family 2. DNA sequencing chromatograms showed that compared with the wild-type control, a novel missense variant, c.871T>C (p.Tyr291His), was identified in proband 2. (c) Pedigree of the affected Family 3. DNA sequencing chromatograms showed that compared with the wild-type control, a novel missense variant, c.644A>C (p.Gln215Pro), was identified in proband 3. (d) Conservation analysis of MSX1 amino acid sequences among different species. Black squares indicate NSTA patients; the black arrow points to the proband; the red arrow indicates the variant site.

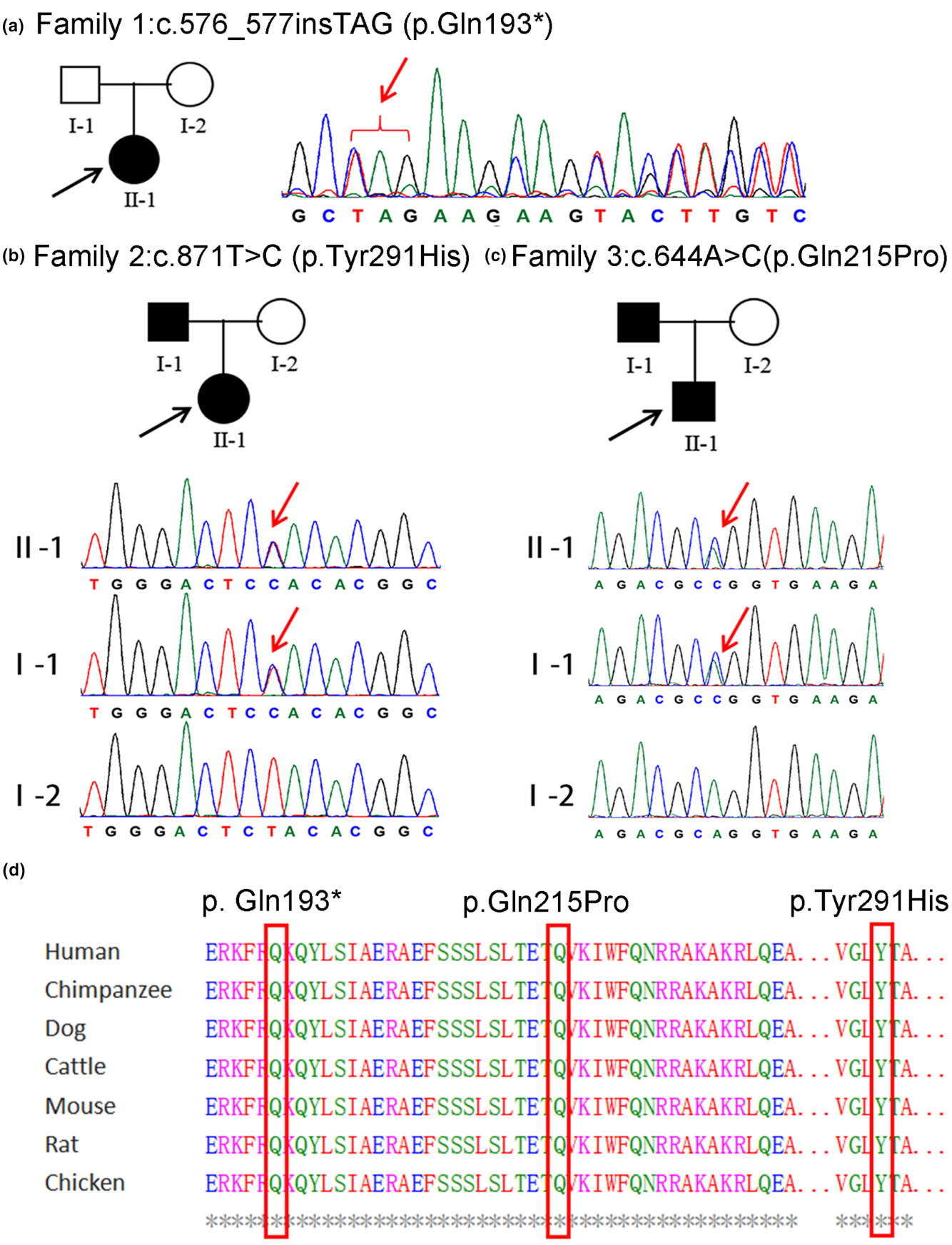
(a) Family 1:c.576_577insTAG (p.Gln193*)
I-1
I-2
II-1
G C T A G A A G A A G T A C T T G T C
(b) Family 2:c.871T>C (p.Tyr291His)
(c) Family 3:c.644A>C(p.Gln215Pro)
I-1
I-2
II-1
II -1
T G G G A C T C C A C A C G G C
I -1
T G G G A C T C C A C A C G G C
I -2
T G G G A C T C T A C A C G G C
I-1
I-2
II-1
II -1
A G A C G C C G G T G A A G A
I -1
A G A C G C C G G T G A A G A
I -2
A G A C G C A G G T G A A G A
(d)
p. Gln193*
p.Gln215Pro
p.Tyr291His
Human
Chimpanzee
Dog
Cattle
Mouse
Rat
Chicken
ERKFRQKQYLSIAERAEFSSSLSLTETQVKIWFQNRRAKAKRLQEA...VGLYTA...
****

TABLE 1 Harm prediction of the three novel *MSX1* variants.

Affected family	Exon	Nucleotide change	Amino acid change	Type of mutation	dbSNP	SIFT	PolyPhen-2	Mutation taster	LRT	ACMG
Family 1	2	c.576_577insTAG	p. Gln193*	Insertion	Novel	—	—	—	—	Pathogenic (PVS1 + PM2 + PP3)
Family 2	2	c.871T>C	p.Tyr291His	Missense	Novel	0.019 Deleterious	1 Probably damaging	1 Disease_causing	0 Deleterious	Uncertain significance (PM2 + PP3)
Family 3	2	c.644A>C	p.Gln215Pro	Missense	Novel	0 Deleterious	0.998 Probably damaging	1 Disease_causing	0 Deleterious	Likely pathogenic (PS2+ PP1 + PP3)

Abbreviations: ACMG, American College of Medical Genetics; PP, supporting evidence of pathogenicity; PS, strong evidence of pathogenicity; PM, moderate evidence of pathogenicity; PVS, very strong evidence of pathogenicity.

Genetic analysis of the Family 2 proband revealed a heterozygous missense variant, c.871T>C; p.Tyr291His, stemming from a heterozygous T>C transition at amino acid position 291, which led to the replacement of tryptophan with histidine at residue 291 (Figure 2b). The proband's father (NSO) carried the variant and the proband's mother was normal at this site, which indicated that the proband inherited the variant from the father and that it was autosomal dominant. This missense variant was predicted to be a potentially pathogenic variant by SIFT, PolyPhen-2, and MutationTaster, with the predicted categories "Deleterious" (0.019), "Probably damaging" (1), and "Disease_causing" (1.00), respectively (Table 1). Sequence analysis identified evolutionary conservation of the involved residue Trp291 among species (Figure 2d).

Another *MSX1* missense variant, c.644A>C; p.Gln215Pro, was detected in the Family 3 proband (Figure 2c). Nucleotide sequencing revealed a heterozygous A>C transition at nucleotide 644, which resulted in the substitution of glutamine to proline at residue 215. The proband's father also carried the variant, and the proband's mother was wild-type at this locus. Sanger sequencing revealed that the missense variant is inherited autosomal dominant from the father. The MSX1 cross-species alignment demonstrated high conservation of Glu215 (Figure 2d).

3.3 | Structural modeling of novel MSX1 variants

The insertion p.Gln193* variant was a truncated protein, and its predicted structure changed significantly compared with the wild-type. The glycine residues at positions 121 and 122 changed from a helical structure to a coil structure (Figure 3b). The two missense variants, p.Tyr291His (Figure 3c) and p.Gln215Pro (Figure 3d), have little structural change compared with the wild-type, with structural changes only in individual amino acid positions affected by the mutations.

We conducted 3D structural analysis to examine the conformational alterations and functional effects of the three novel *MSX1* variants. The conformation of wild-type MSX1 at positions 192 and 215 was predicted (Figure 3e,g). In comparison with the wild-type, the p.Gln193* variant resulted in the absence of the third helix behind the 192nd position and termination of the protein to form the truncated variant. The number of hydrogen bonds around the 192nd residue was decreased, and the hydrogen bonds became longer (Figure 3f).

p.Gln215 of wild-type MSX1 was connected to the surrounding amino acids via four hydrogen bonds (Figure 3g). However, in the p.Gln215Pro variant (Figure 3h), the hydrophilicity of the amino acid residue decreased, and the

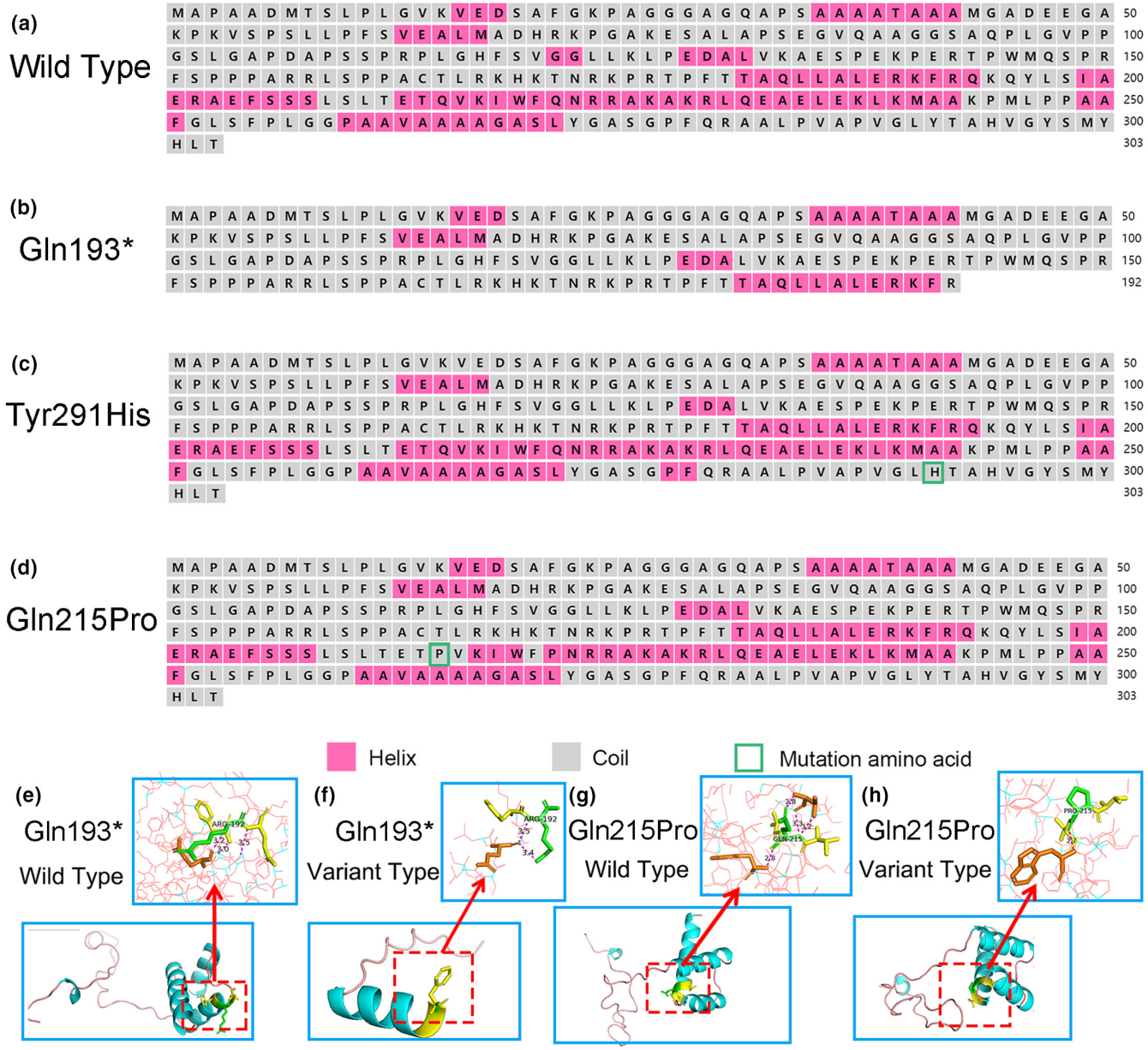

FIGURE 3 Secondary structure analysis and three-dimensional protein structural modeling of the wild-type and variant type MSX1 proteins. (a–d) The predicted secondary structure of the wild-type and variant MSX1 proteins. The pink square represents α-helix, the gray square represents β-coil, and the green square represents the variant amino acid. (e, f) Comparison of three-dimensional structure between the wild-type and the p.Gln193* variant. (g, h) Comparison of three-dimensional structure between the wild-type and the Gln215Pro variant. The green stick-like structure shows the mutated amino acid. The red arrow indicates that the target area was enlarged.

side-chain was replaced by a secondary amine, leading to an increase in volume. Furthermore, the variant caused the absence of the three hydrogen bonds with Thr212 and the shortening of the hydrogen bond with Trp219.

Unfortunately, we were unable to construct a model of the variant p.Tyr291His at the protein terminus.

Our 3D structural analysis results indicated that the changes in protein structure caused by the *MSX1* variants might change the interaction(s) between MSX1 and other crucial tooth development regulatory signaling molecules.

3.4 | Novel variant of MSX1 p.Gln193* influences MSX1 protein nuclear localization

We assessed whether the nuclear localization of the expressed protein was affected. MSX1 is normally located in the nucleus (Zheng et al., 2021). Immunofluorescence showed that the Gln193* variant was present in the cytoplasm of transiently transfected hDPSCs (Figure 4). The insertion variant of Gln193* impairs nuclear localization of MSX1.

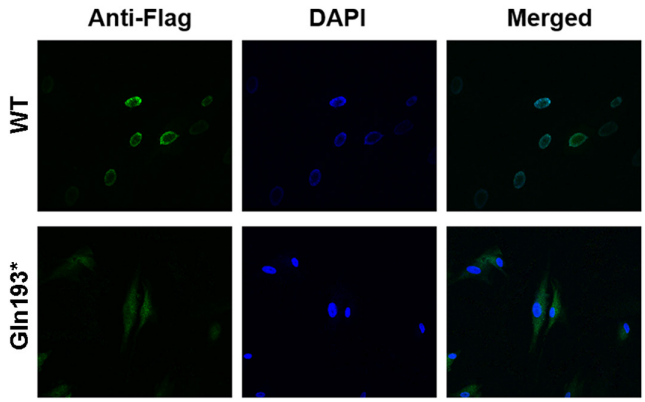

FIGURE 4 Subcellular localization of wild-type and variant MSX1 proteins in human dental pulp stem cells. Blue indicates nuclear staining by 4′,6′-diamidino-2-phenylindole (DAPI), and green indicates anti-FLAG labeling. Wild-type MSX1 was found only in the nucleus, while the Gln193* variant was distributed in the cytoplasm.

3.5 | Genotype–phenotype analysis of NSTA patients with *MSX1* variants

We summarized and analyzed the genotype–phenotype relationships of NSTA caused by *MSX1* variants reported in 1996–2022 (Abid et al., 2017; Adachi et al., 2021; AlFawaz et al., 2015; Arte et al., 2013; Bergendal et al., 2011; Biedziak et al., 2022; Bonczek et al., 2018; Ceyhan et al., 2014; Chishti et al., 2006; Daw et al., 2017; De Muynck et al., 2004; Kamamoto et al., 2011; Keskin et al., 2022; Kim et al., 2006; Kimura et al., 2014; Ma et al., 2020; Mitsui et al., 2016; Mostowska et al., 2006, 2012; Mu et al., 2013; Şahan & Akan, 2021; Tatematsu et al., 2015; Vastardis et al., 1996; Wong et al., 2014; Xin et al., 2018; Xuan et al., 2008; Xue et al., 2016; Yamaguchi et al., 2014; Yang et al., 2020; Yue et al., 2022; Zheng et al., 2021). In brief, we identified 46 patients with 44 *MSX1* variants, including the patients in this study (Table S1). *MSX1*-related missense variants comprised 50% of the 44 variants (Figure 5a). *MSX1*-related variants are most likely to occur in the conserved HD of exon 2 (Figure S1). Assessment of the relationship of the *MSX1*-related phenotype of NSTA ($n = 46$) determined it is possible for all types of permanent teeth to be missing, with a left–right symmetry trend. In descending order, the teeth most liable to be congenitally absent (>50%) were second premolars, 15 (89.1%) > 25, 35 (84.8%) > 45 (82.6%), lower-right-upper first premolars (76.1%), and left-upper-first premolars (69.6%). The most infrequently lost teeth were canines, which were both lost in only 8.7% of cases (Figure 5b and Table S2).

4 | DISCUSSION

Tooth development depends upon a consecutive and shared series of inductive events involving coordination of conserved signaling pathways, which include those for FGF, BMP, WNT, and SHH signaling, occurring between the epithelium and the mesenchyme derived from the neural crest (Bei, 2009). According to Yu et al., variants in the following seven genes cause around 91.9% of non-syndromic congenital tooth loss: *WNT10A*, *AXIN2*, *PAX9*, *LRP6*, *EDA*, *WNT10B*, and *MSX1* (Yu et al., 2019).

MSX1 variants lead to NSTA (Yang et al., 2020) and exhibit autosomal dominant genetic inheritance (Wong et al., 2014). Zheng et al. (2021) summarized the discovery of *MSX1* variants related to NSTA through 2018, and found that the HD contains 75% of missense variants, and only 33% of frameshift variants. In the present study, we identified three novel pathogenic variants of *MSX1* in 35 unrelated Chinese families that had NSO. The three novel *MSX1* variants included one insertion variant (c.576_577insTAG, p. Gln193*) and two missense variants (c.871T>C,p.Tyr291His; c.644A>C, p.Gln215Pro). All variants were in *MSX1* exon 2, and the Gln193* and Gln215Pro variants were located in the HD region. As far as we know, these three variants have not been reported previously. Protein sequence analysis showed that residues Gln193, Gln215, and His291 were highly evolutionarily conserved. Thus, *MSX1* variants are more liable to be found in evolutionarily conserved parts of the protein.

A highly conserved sequence, the HD comprises an extended *N*-terminal arm (No. 172–180) and three α-helices (helix I: No. 181–193, helix II: No. 199–209, and helix III: No. 213–231), which contribute to the stability of the structure, the specificity of DNA binding, repression of transcription, and protein–protein interactions of MSX1 (Isaac et al., 1995; Yang et al., 2020). Through 3D reconstruction of the MSX1 protein structure, we found that p. Gln193* is at the end of the first α-helix, resulting in a marked truncation of the protein in the HD region. In comparison with the wild-type, the Gln215Pro variant causes a polar glutamine residue to change into a non-polar proline residue, replacing the side-chain by a secondary amine, and leading to an increase in volume. The Gln215Pro variant is near the start of the third α-helix, changing the shape of this helix and affecting its interaction with surrounding amino acid residues. Yang et al. (2020) reported that helixes I and II are closely related to structural stability, while helix III is very important for the specific binding of MSX1 to DNA. Both p. Gln193* and p.Gln215Pro altered the structure of helix III. Therefore, we speculate that this novel Gln193* insertion variant and the Gln215Pro seriously impact MSX1 protein–protein interactions and DNA binding.

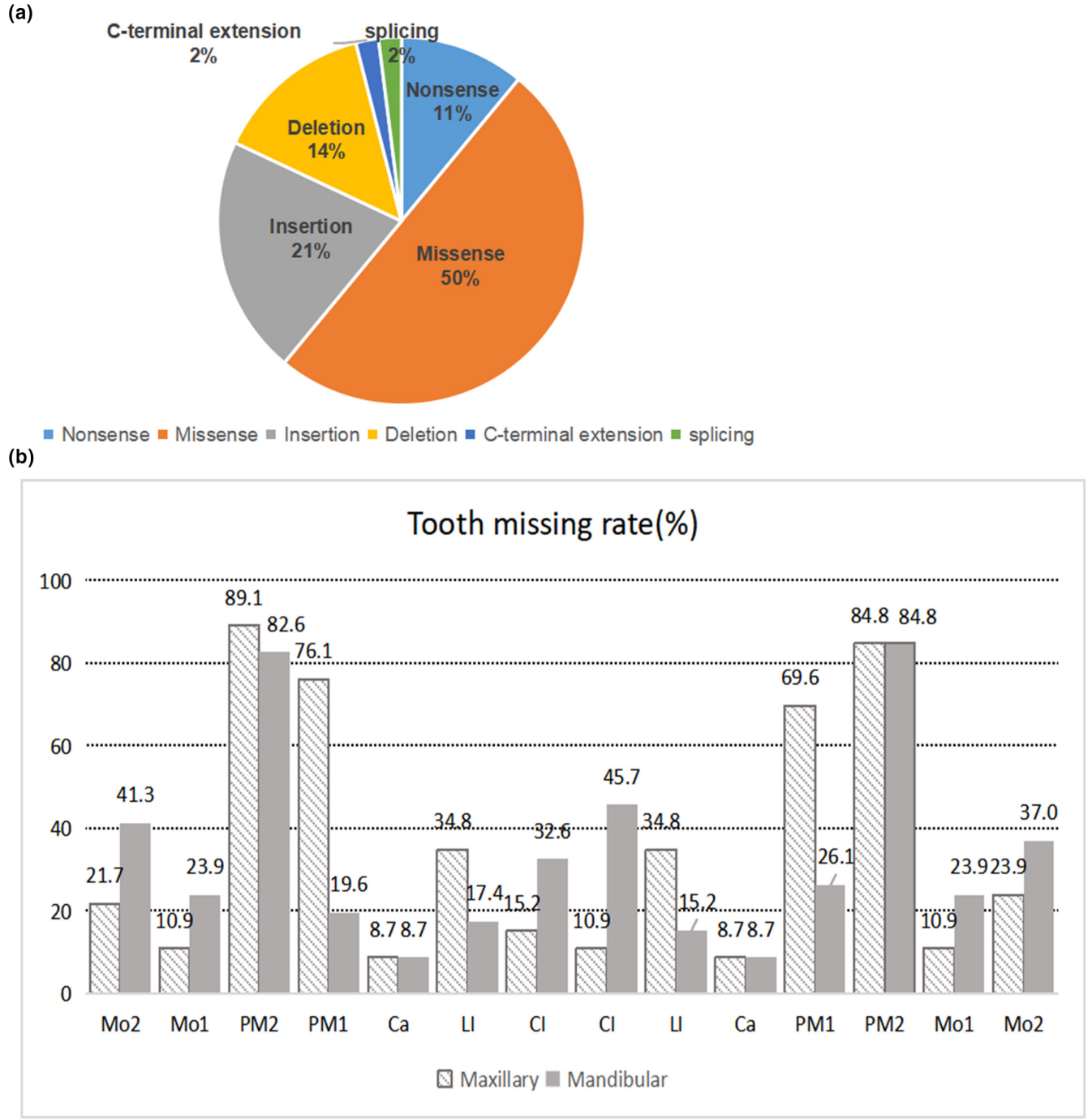

FIGURE 5 Genotype–phenotype analysis of *MSX1* variants. (a) Proportion of *MSX1* variant types; (b) Missing tooth rate of patients with NSTA (excluding the third molars) associated with *MSX1* variants ($n = 46$). Mo2, second molar; Mo1, first molar; PM2, second premolar; PM1, first premolar; Ca, canine; LI, lateral incisor; CI, central incisor.

Proteins fold into specific conformations chiefly through non-covalent interactions, which include ionic bonds, hydrogen bonds, hydrophobic interactions, and van der Waals forces (Muñoz et al., 1996). Hydrogen bonding is crucial in the formation of the secondary structure and 3D structural integrity (Wang et al., 2006; Yang et al., 2020). In this study, through 3D reconstruction of MSX1, we determined that, in comparison with the wild-type protein, the Gln215Pro variant exhibits decreased hydrophilicity of the amino acid residue, leading to changes in configuration. Furthermore, this variation leads to the disappearance of the three hydrogen bonds with residue Thr212 and shortening of the hydrogen bond to Trp219. We were unable to construct a structural model the p.Tyr291His variant, so we were could not predict the change in 3D conformation resulting from this variant.

MSX1 is a transcription factor; the *MSX1* HD (amino acids 175–229) is decisive in molecular interactions with DNA (Hovde et al., 2001) and is associated with nuclear transport (Wang et al., 2011). The HD region contains two nuclear localization signal (NLS) sequences: NLS1 (^{161}RKHKTNRKPR^{170}) upstream of the HD, and NLS2 (^{216}NRRAKAKR^{223}). NLS1 and NLS2 cooperate to facilitate MSX1 nuclear localization (Shibata et al., 2018). Therefore, the presence or absence of mutations in the HD might lead to impaired or lost MSX1 nuclear localization (Zheng et al., 2021). We found that wild-type MSX1 protein localized to the nucleus, but the Gln193* variant protein with significant changes to the structure in the HD area was mostly localized to the cytoplasm, which is consistent with previous reports that ectopic expression of a truncated *MSX1* variant resulted in altered subcellular localization of the MSX1 protein, indicating that this mutation seriously affects the nuclear translocation of the transcription factor (Adachi et al., 2021; Tatematsu et al., 2015; Wong et al., 2014; Xin et al., 2018; Zheng et al., 2021). We postulate that changes in the subcellular localization of MSX1 Gln193* may directly cause tooth oligodontia.

To confirm the *MSX1*-related tooth agenesis phenotype features in detail, we evaluated 46 cases from published articles and the three Chinese Han pedigrees identified in this study. Phenotypic analysis revealed that *MSX1* variants associated with NSTA were most likely to cause the loss of second premolars (excluding the third molar), with the right maxillary second premolar being the most commonly missing second premolar (89.1%). The canine was the least likely tooth to be lost (8.7%). In this study, all the second premolars of the probands were missing, which is consistent with the missing tooth characteristics of *MSX1* variants, and indicates that *MSX1* is very important for the development of premolars. Liang et al. (2016) found that missense variants of *MSX1* were more frequent than truncated variants, and pointed out that alterations to the HD domain caused great damage to *MSX1* function as a repressor of transcription, resulting in different phenotypes. Also, we discovered that the majority (50%) of the *MSX1* variations were missense variants that were clustered in the HD area on exon 2, confirming the significance of the HD region structure. The findings of our study are in agreement with previous research (Alkhatib et al., 2022; Liang et al., 2016; Zheng et al., 2021).

5 | CONCLUSIONS

We identified three new *MSX1* variants in three unrelated Chinese NSO families: one insertion variant (c.576_577insTAG, p. Gln193*), and two missense variants (c.871T>C, p.Tyr291His and c.644A>C, p.Gln215Pro). These three variants were forecast to be pathogenic, and genotypic and phenotypic results suggest that these *MSX1* variants result in the loss of the second premolars. Our study expands the *MSX1* variant spectrum, and presents a genetic origin for the pathogenesis observed in these patients and their families.

AUTHOR CONTRIBUTIONS

Ya Zhao was involved in conceptualization, data curation, formal analysis, methodology, and writing—original draft. Jiabao Ren was involved in data curation, formal analysis, and writing—review and editing. Lingqiang Meng was involved in investigation and validation. Yan Hou and Chunyan Liu were involved in conceptualization and software. Guozhong Zhang was involved in software and resources. Wenjing Shen was involved in data curation, acquisition, supervision, and writing—review and editing. All author read and approved the final version of the manuscript.

ACKNOWLEDGMENTS

We thank all study participants for their contributions. This manuscript has also been proofread by an English-speaking professional with a science background at Elixigen Corporation.

FUNDING INFORMATION

This work was supported by the Natural Science Foundation of Hebei Province, China (grant number H2022206246); the Special Project of Health Innovation of Hebei Provincial Department of Science and Technology of China (grant number 21377716D); and the Hebei Provincial Government Funded Clinical Medicine Talents Training and Basic Research Projects (grant number 361029).

CONFLICT OF INTEREST STATEMENT

The authors have no potential or existing conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All the research procedures performed in this study were in accordance with the ethical standards of the institutional and / or national research committee and with the 1964 World Medical Association Declaration of Helsinki and its

later amendments. This study was approved by the Ethics Committee of the School and Hospital of Stomatology, Hebei Medical University (NO: [2016] 004) and written informed consent was obtained for all participants.

CONSENT TO PARTICIPATE

Written informed consent was obtained from the [individual(s) AND/OR minor(s)' legal guardian/next of kin] for the publication of any potentially identifiable images or data included in this article.

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How to cite this article: Zhao, Y., Ren, J., Meng, L., Hou, Y., Liu, C., Zhang, G., & Shen, W. (2024). Characterization of novel *MSX1* variants causally associated with non-syndromic oligodontia in Chinese families. *Molecular Genetics & Genomic Medicine*, *12*, e2334. https://doi.org/10.1002/mgg3.2334