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Novel *PITX2* mutations identified in Axenfeld–Rieger syndrome and the pattern of *PITX2*-related tooth agenesis

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Abstract

Objectives: To investigate the mutations in patients with Axenfeld–Rieger syndrome (ARS) and the pattern of *PITX2*-related tooth agenesis.

Methods: Whole-exome sequencing (WES) and copy number variation (CNV) array were used to screen the mutations in four ARS probands. After Sanger sequencing and quantitative polymerase chain reaction (qPCR) validation, secondary structure prediction and dual-luciferase assay were employed to investigate the functional impact. Eighteen *PITX2*-mutated patients with definite dental records were retrieved from our database and literatures, and the pattern of *PITX2*-related tooth agenesis was analyzed.

Results: A novel de novo segmental deletion of chromosome 4q25 (GRCh37/hg19 chr4:111, 320, 052–111, 754, 236) encompassing *PITX2* and three novel *PITX2* mutations c.148C > T, c.257G > A, and c.630insCG were identified. Preliminary functional studies indicated the transactivation capacity of mutant PITX2 on *Distal-less homeobox 2* (*DLX2*) promoter was compromised. The maxillary teeth showed significantly higher rate of agenesis (57.94%) than the mandibular teeth (44.05%). The most often missing teeth were upper lateral incisors (83.33%) and upper second premolars (69.44%). Teeth with the least agenesis rate were the lower second molars (19.44%) and lower first molars (8.33%).

Conclusions: We identified a novel 4q25 microdeletion including *PITX2* and three novel *PITX2* mutations, and statistically analyzed the *PITX2*-related tooth agenesis pattern.

KEYWORDS
chromosomal microdeletion, ocular malformation, *PITX2* mutation, *PITX2*-related tooth agenesis pattern

Fan, Sun and Liu contributed equally to this work.

1 | INTRODUCTION

Axenfeld–Rieger syndrome (ARS; MIM #601499, MIM #180500, and MIM #602482) is a clinically and genetically heterogeneous group of developmental disorders and inherited in an autosomal-dominant manner. The typical clinical manifestations of ARS include ocular anterior chamber anomalies, umbilical stump abnormalities, craniofacial malformation, and dental anomaly (Seifi & Walter, 2018). The craniofacial dysmorphism of ARS involves a wide spectrum of developmental dysplasia, including maxillary hypoplasia, prominent forehead, telecanthus, hypertelorism, and a flattened midface with flat nasal bridge (O'Dwyer & Jones, 2005; Tumer & Bach-Holm, 2009). Typical ocular defects include bilateral iris hypoplasia, iridocorneal adhesion, polycoria, corectopia, and posterior embryotoxon caused by anterior displacement of Schwalbe's line (Kaminska, Sokolowska-Oracz, Pawluczyk-Dyjecinska, & Szaflik, 2007; Zamora & Salini, 2019). Phenotypes related to dental anomaly usually include tooth agenesis and/or tooth shape abnormity, such as microdontia and conical teeth (Jena & Kharbanda, 2005; O'Dwyer & Jones, 2005).

Traditional genetic studies and current advances in molecular genetics have identified two major ARS genes, *paired-like homeodomain transcription factor 2* (*PITX2*; 4q25; OMIM *601542) and *forkhead box C1* (*FOXC1*; 6p25; OMIM *601090). Various types of mutations in *PITX2* and *FOXC1* are found in patients with ARS, including small point mutations, insertion mutations, and chromosomal deletions (Seifi & Walter, 2018). Unlike *FOXC1* mutations are commonly found in ARS patients with sensorineural hearing loss and cardiac abnormalities, *PITX2* mutations are more frequently detected in ARS patients with dental anomaly, ocular dysplasia, and umbilical anomalies (Hendee et al., 2018). More recently, *PITX2* mutation is also associated with non-syndromic tooth agenesis (Intarak et al., 2018).

As a transcription factor, PITX2 plays an important role during embryonic development, especially in pattern formation, left-right asymmetry, cell differentiation, and apoptosis (Gage, Suh, & Camper, 1999; Matalova, Fleischmannova, Sharpe, & Tucker, 2008). In murine tooth development, the expression of *Pitx2* is restricted to the dental epithelium throughout odontogenesis and is detectable before the tooth buds initiate from embryonic day 8 (St Amand et al., 2000). Because of the critical function in tooth development, knockout of *Pitx2* in mice results in the tooth germ arrested at bud stage (Lin et al., 1999). Although the exact pathogenic mechanisms remain unknown, one possible explanation of ARS-associated tooth agenesis appears to be that the PITX2 mutant is unable to activate tooth morphogenesis-related genes, such as *Distal-less homeobox 2* (*DLX2*; 2q31.1; OMIM *126255) (Espinoza, Cox, Semina, & Amendt, 2002).

Although many mutations in *PITX2* have been identified in ARS and non-syndromic tooth agenesis individuals, the pattern of *PITX2*-associated tooth agenesis has not been systematically investigated. In this study, we identified four novel *PITX2* mutations, including a heterozygous chromosome 4q25 deletion encompassing *PITX2*, two nonsense mutations, and one frameshift mutation, and investigated the impact of the nonsense and frameshift mutations on *PITX2* function by preliminary functional studies. Furthermore, we described the detailed clinical features including the tooth agenesis positions and the phenotype variance of ophthalmic defect in our patients with ARS. Finally, we summarized the missing tooth positions of *PITX2*-mutated patients including ARS and non-syndromic tooth agenesis and statistically analyzed the *PITX2*-related tooth agenesis pattern.

2 | MATERIALS AND METHODS

2.1 | Studied individuals

Four individuals from four unrelated families with a clinical diagnosis of ARS were recruited from the Department of Prosthodontics in the Peking University Hospital of Stomatology, Beijing, China. None of the individuals reported the history of tooth extraction or tooth loss. Informed consents were signed by all participants. Intra-oral, ophthalmologic, and radiographic examinations were performed. This study was approved by the Ethics Committee of Peking University School and Hospital of Stomatology (PKUSSIRB-201736082).

2.2 | DNA sequencing and mutational analysis

Genomic DNA samples were isolated from peripheral blood lymphocytes or saliva using the Blood Genomic DNA Mini Kit (Cwbiotech) or the ORAgene-DNA Kit (ORAGENE), according to the manufacturer's protocols. The probands' genomic DNA samples extracted from peripheral blood lymphocytes were sent for WES and CNV array (iGeneTech) to identify potential pathogenic mutations through the Illumina X10 sequencing platform. And the variants were filtered based on following strategies: (a) The genes included in the orodental-related gene list were analyzed (Prasad et al., 2016); (b) then, silent mutations and missense variants with a minor allele frequency (MAF) ≥0.01 in East Asians in the 1,000 Genomes Project (1000G, http://www.1000genomes.org), Exome Aggregation Consortium (ExAC, http://exac.broadinstitute.org), Genome Aggregation Database (gnomAD, http://gnomad.broadinstitute.org/), or Single Nucleotide Polymorphism database (dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/snp_summary.cgi/) were excluded; and (c) bioinformatics analysis was used for the remaining missense variants to predict the functional impact by Mutation Taster (http://www.mutationtaster.org), Polymorphism Phenotyping v2 (PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/), and Sorting Intolerant from Tolerant (SIFT, https://sift.bii.a-star.edu.sg/). After that, for mutation validation and family co-segregation, the coding regions of human *PITX2* (NM_153427.2) were amplified by polymerase chain reaction (PCR; see primer sequences in Table S1), the amplified PCR products were sequenced by Tsingke Biological and the results were blasted on NCBI. Genomic quantitative polymerase chain reaction (qPCR; see primer sequences in Table S2) analysis was used to confirm CNVs, as previously described (Yang, Wang, Zhao, & Qin, 2018).

TABLE 1 Summary of genotype and phenotype observed in patients in this study

Proband Number	Age and Gender	Mutational analysis	Exon	Mutation type	ACMG Classification (evidence of pathogenicity)	Clinical manifestation				
						Maxillary hypo-plasia	Umbilical stump anomaly	Ocular anomaly	Number of missing permanent teeth	Number of missing primary teeth
#607 Proband	21/Female	0.43 Mb deletion chr4:111320052–111754236)	All	CNV	Pathogenic (PVS1 + PS2+ PP4)	Yes	Yes	Corneal opacity, iridocorneal adhesion, angle closure	14	Unknown
#577 Proband	29/Female	c.148C > T; p.Q50X	4	Nonsense	Pathogenic (PVS1 + PS2+PS3 + PM2 +PP1 + PP4)	Yes	Yes	Posterior embryotoxon, iris hypoplasia, corectopia, iridocorneal adhesion	13	Unknown
#120 Proband	20/Female	c.257G > A; p.W86X	5	Nonsense	Pathogenic (PVS1 + PS2+PS3 + PM2+PP4)	Yes	Yes	Severe glaucoma (details unknown)	26	Unknown
#535 Proband	7/Female	c.630insCG; p.V211RfsX28	5	Frameshift	Pathogenic (PVS1 + PS2+PS3 + PM2+PP4)	Yes	Yes	slight corectopia	23	9

Abbreviations: ACMG, American College of Medical Genetics; PM, pathogenic criterion is weighted as moderate; PP, pathogenic criterion is weighted as supporting; PS, pathogenic criterion is weighted as strong; PVS, pathogenic criterion is weighted as very strong.

Structural changes of PITX2 mutant were predicted using PsiPred 3.3 (http://bioinf.cs.ucl.ac.uk/psipred).

2.3 | Construction of the expression and reporter plasmids

The full-length coding sequence of wild-type PITX2 (NP_700476.1; WT) was subcloned into pEGFP-C1 vector (empty vector). PITX2 mutant plasmids, pEGFP-PITX2 (W86X), pEGFP-PITX2 (Q50X), and pEGFP-PITX2 (V211RfsX28) were constructed as previously described (Lee, Shin, Ryu, Kim, & Ryu, 2010). The *DLX2* reporter plasmid with firefly luciferase (pGL3-DLX2) was constructed as previously described (Espinoza et al., 2005). All plasmids were confirmed by DNA sequencing (Tsingke Biological).

2.4 | Cell culture, transient transfection, and luciferase assay

Because there was no human odontogenic cell line available commercially, we chose human embryonic kidney 293T cell line which was widely used for dual-luciferase reporter assay and a murine dental epithelial cell line LS8 to perform the functional analysis. Two cell lines were seeded in 24-well plates at a density of 1.5×10^5 per well, respectively, and cultured in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin–streptomycin (Solarbio) at 37°C with 5% CO_2 and 95% air. Each PITX2 expression plasmid and empty vector were co-transfected with pGL3-DLX2 and phRL-TK Renilla luciferase vector. After 24-hr transfection, cell lysates were collected and both Firefly and Renilla luciferase activities were detected through a dual-luciferase reporter assay system (Promega) and a Veritas™ Microplate Luminometer (Turner Biosystems). Independent luciferase reporter experiments were carried out in triplicate. The fold activation of Firefly luciferase normalized to Renilla luciferase was used for detecting the transcriptional activity of the *DLX2*.

2.5 | Statistical analysis

In order to analyze the tooth agenesis pattern in patients with *PITX2* mutations, eight patients with detailed tooth agenesis sites records were from our own database and previously published work (Wang, Zhao, Zhang, & Feng, 2003). We searched in PubMed with keyword "Tooth" and "PITX2", and found 86 literatures. After the careful reading, only 10 patients with detailed documentation of tooth agenesis sites (or with panoramic films) and *PITX2* variation were found from six literatures. Therefore, 18 patients were included for analysis totally, and none of them had the history of tooth extraction or tooth loss.

Statistical analysis was performed using SPSS 20.0 (SPSS, Inc.). For dual-luciferase reporter assay and rate of tooth agenesis, one-way analysis of variance and Chi-square (or Fisher's exact) test were performed to compare the differences between groups. Data were

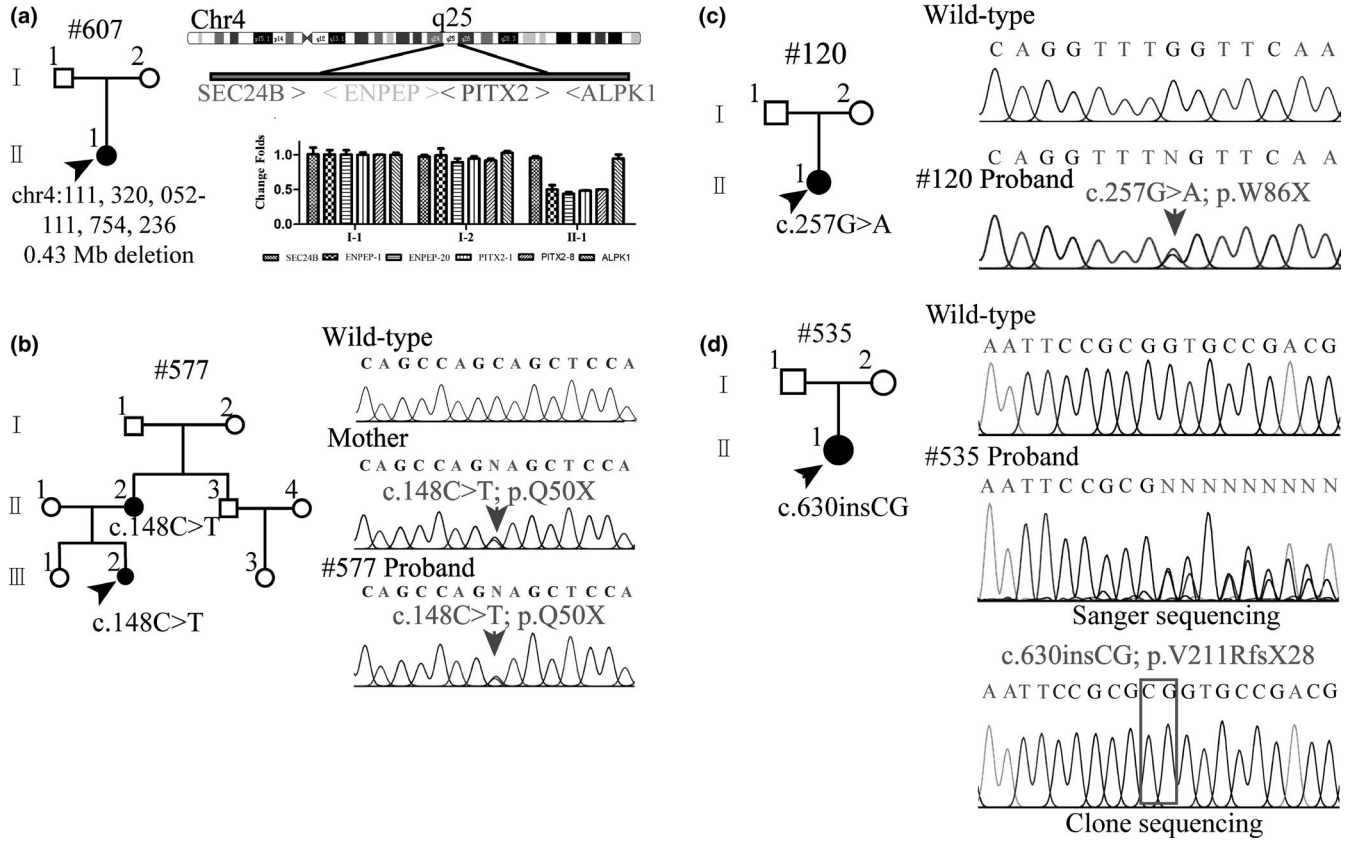


FIGURE 1 Pedigree and *PITX2* mutations. (a) In the #607 proband, CNV array and qPCR results indicated *ENPEP* and *PITX2* genome DNA were only half to normal control. (b) In the #577 proband and her mother (II-2), a novel *PITX2* nonsense mutation (NM_153427.2, c.148C > T; p.Q50X) was detected. (c) In the #120 proband, a novel *PITX2* nonsense mutation (NM_153427.2, c.257G > A; p.W86X) was found. (d) In the #535 proband, a novel *PITX2* frameshift mutation (NM_153427.2, c.630insCG; p.V211RfsX28) was identified [Colour figure can be viewed at wileyonlinelibrary.com]

presented as mean ± *SD* (n = 3) with a $p < .05$ considered as statistically significant.

3 | RESULTS

Based on the results of orofacial and ophthalmologic examinations, four patients clinically diagnosed as ARS were studied. WES, CNV array, and Sanger sequencing revealed a novel de novo segmental deletion of chromosome 4q25 (GRCh37/hg19 chr4:111, 320, 052–111, 754, 236) encompassing *PITX2* and three novel *PITX2* mutations. (All data are available at the NCBI SRA database: www.ncbi.nlm.nih.gov/sra/ under Accession Number PRJNA561511). The clinical characteristics and the novel mutations of *PITX2* were summarized in Table 1.

3.1 | Mutational analysis and clinical findings

#607 proband was a 21-year-old woman carrying a novel heterozygous chromosomal deletion on 4q25 (GRCh37/hg19 chr4:111, 320, 052–111, 754, 236) encompassing *PITX2* that was identified by WES and CNV array (Figure 1a). She had agenesis of 14 permanent teeth, which included all her maxillary anterior teeth and some of her premolars and molars (Figure 2a,b,c). The umbilical stump abnormality was observed (Figure 2d). Moreover, ophthalmologic examinations demonstrated that she had severe ocular anterior chamber anomalies, including corneal opacity, iridocorneal adhesion, and angle closure (Figure 2e,f). The qPCR results confirmed that the expression of *PITX2* and *ENPEP* was eliminated in one of her chromosomes 4 (Figure 1a). No dental, umbilical, ocular abnormalities or *PITX2* mutations were found in the proband's parents, indicating that the mutation of #607 proband was de novo.

A novel nonsense *PITX2* mutation (c.148C > T; p.Q50X) was detected in a 29-year-old woman (#577 proband) (Figure 1b), who had agenesis of 13 permanent teeth, including most of the anterior teeth and right mandibular second premolar (Figure 2g,h,i). The umbilical stump anomaly was observed (Figure 2j). Furthermore, ophthalmologic examinations demonstrated that she had severe ocular anterior chamber anomalies, including posterior embryotoxon caused by anterior displacement of Schwalbe's line, iris hypoplasia, iridocorneal adhesion, and corectopia (Figure 2k,l). Similarly, her mother showed typical ARS features and had been edentulous for many years with tooth extraction history; however, the photographs of her phenotype and detailed tooth agenesis positions were not available. The

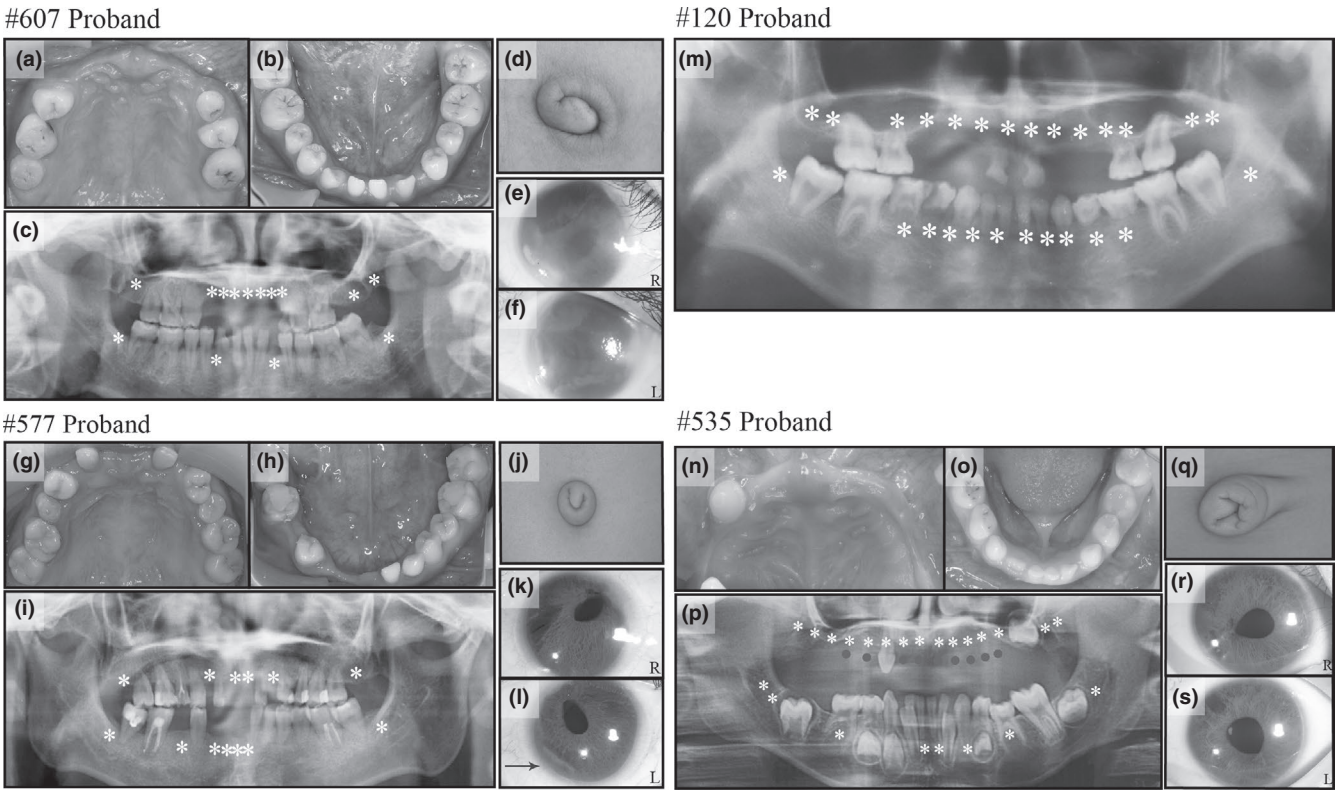


FIGURE 2 Clinical features of probands. Intra-oral photographs of proband #607 (a and b), #577 (g and h), and #535 (n and o). (c, i, m, and p) Panoramic radiographs of four probands. (d, j, and q) Umbilical photographs of proband #607, #577, and #535. Ocular symptoms of the proband #607 (e and f), #577 (k and l), and #535 (r and s). White asterisks indicate congenital missing permanent teeth. Red circles indicate congenital missing primary teeth. R, right side; L, left side [Colour figure can be viewed at wileyonlinelibrary.com]

proband's father and sister were unaffected. Genetic analysis indicated that the proband's nonsense *PITX2* mutation was inherited from her mother (Figure 1b).

#120 proband was a 20-year-old woman carrying a novel *PITX2* nonsense mutation (c.257G > A; p.W86X) (Figure 1c). Excluding tooth extraction, she had agenesis of 26 permanent teeth, and 14 deciduous teeth were retained (Figure 2m). Almost all of her permanent teeth were absent, except the first molars and mandibular second molars. The umbilical stump anomaly was observed. In addition, she was diagnosed as glaucoma and caused complete permanent blindness many years ago, and the detailed ophthalmologic examination information was not available. No dental, umbilical, ocular abnormalities or *PITX2* mutations were found in her parents, indicating that the nonsense mutation of #120 proband was de novo.

#535 proband was a 7-year-old girl who carried a novel *PITX2* frameshift mutation (c.630insCG; p.V211RfsX28) (Figure 1d). The CG insertion located in the transcriptional activation domain 2 (TAD2) domain of PITX2, resulted in a premature termination at amino acid 239. Excluding tooth extraction, she had agenesis of 23 permanent teeth, including almost all her maxillary teeth except left first molar and some of her mandibular incisors, premolars, and molars (Figure 2n,o,p). Interestingly, she only had a right deciduous canine in maxilla, but all her deciduous teeth were present in mandible (Figure 2n,o,p). The typical umbilical stump abnormality was identified (Figure 2q). It is noteworthy that her ocular anterior chamber anomaly was much milder than the other probands mentioned above, and only iris hypoplasia and slight corectopia were observed (Figure 2r,s). No abnormalities or *PITX2* mutations were detected in her parents, indicating that the frameshift mutation of #535 proband was de novo.

3.2 | Functional analyses of *PITX2* mutations

After identifying the novel *PITX2* mutations in patients with ARS, we conducted a bioinformatics analysis to predict the functional effects of *PITX2* nonsense and frameshift mutations. Secondary protein structure prediction showed that wild-type PITX2 (NP_700476.1) was composed of five α-helices and a strand that contains homeodomain (HD) and otp-aristaless-rax homology (OAR) domain (Figure 3a). The two nonsense mutations, p.Q50X and p.W86X, resulted in a truncation of PITX2 in the homeodomain, leaving the N-termination of PITX2 only (Figure 3b,c). The frameshift mutation p.V211RfsX28 changed the C-terminational structure of PITX2 and led to an α-helix appearing in advance (Figure 3d). This mutation was located before OAR domain and resulted in a premature termination of PITX2 at amino acid 239 (Figure 3d).

To further confirm the functional impacts of *PITX2* mutations, we investigated the activation of *DLX2*, a downstream target of *PITX2* in tooth development. The dual-luciferase reporter assay results from human embryonic kidney 293T and murine ameloblast-like LS8 cell

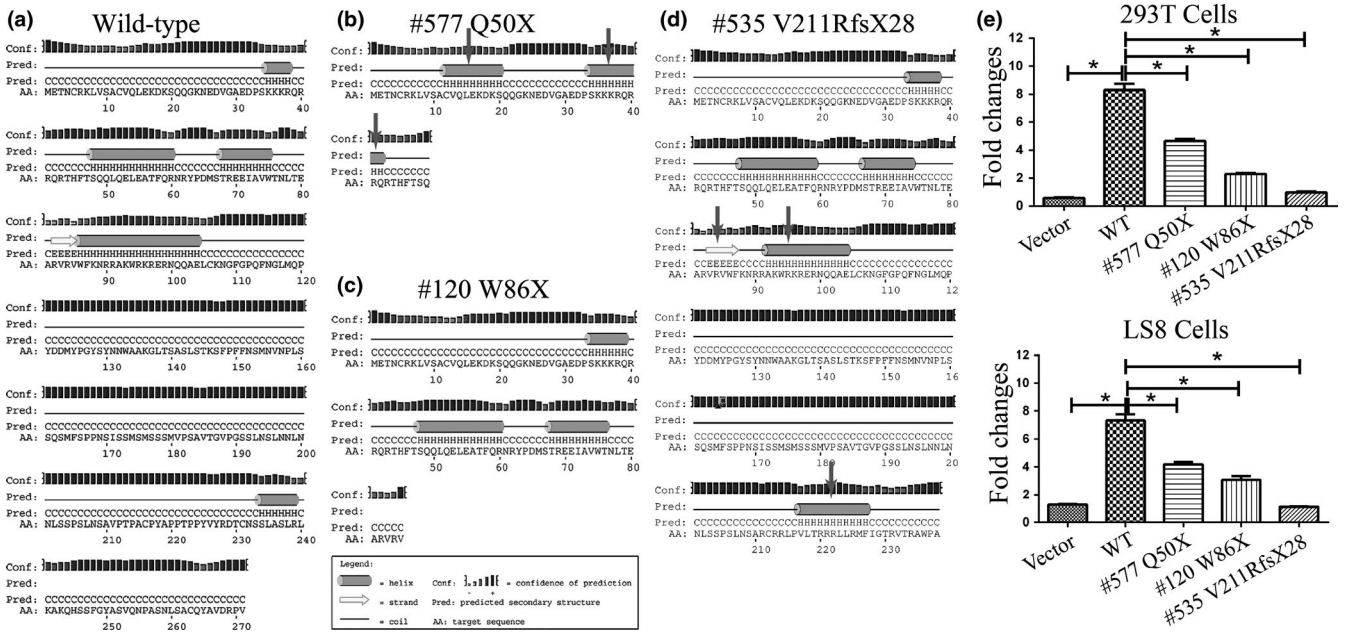


FIGURE 3 Secondary structure prediction and dual-luciferase assay results of PITX2 mutants. (a, b, c, and d) Secondary structure analysis of wild-type and mutated PITX2. The helix structures are presented as pink cylinders, the strands are marked with yellow arrows, and the coils are drawn in straight lines. Transformations have been marked with red arrows. (e) Dual-luciferase assay results of *DLX2*-transactivation capacity of PITX2 mutants in 293T and LS8 cells. *DLX2*-transactivation was reduced by PITX2 mutations. The asterisk denotes the difference with statistical significance (*$p < .05$) [Colour figure can be viewed at wileyonlinelibrary.com]

lines indicated that the activation capacity to *DLX2* was significantly reduced in p.Q50X, p.W86X, and p.V211RfsX28 PITX2 mutants, when compared to which of wild-type PITX2 ($p < .05$) (Figure 3e).

3.3 | Statistical analysis of *PITX2*-related tooth agenesis pattern

Although many *PITX2* mutations are reported, the pattern of *PITX2*-related tooth agenesis remains unknown. Therefore, we analyzed the permanent tooth agenesis positions from a total of 18 *PITX2*-mutated patients with detailed dental records. As described in materials and methods, eight patients with ARS were from our own database, including four probands in this study, one case with previously reported mutation (our unpublished data, patient No.5), and three patients from one family (patient No. 6–8) (Wang et al., 2003). The other eight patients with ARS and two non-syndromic tooth agenesis patients were collected from literatures (patient No.9–18) (Dressler et al., 2010; Idrees et al., 2006; Intarak et al., 2018; Kimura et al., 2014; Meyer-Marcotty, Weisschuh, Dressler, Hartmann, & Stellzig-Eisenhauer, 2008; Yang et al., 2018). Detailed information of missing tooth positions was shown in Table 2.

After statistical analysis, we found that the average number of *PITX2*-related tooth agenesis was 14.28 ± 7.04 (excluding third molars). The average incidence rate of tooth agenesis in maxilla (57.94%) was significantly higher than which in mandible (44.05%) ($p < .05$), indicating that the maxillary teeth were more affected in *PITX2*-mutated patients. The rate of tooth agenesis in left side (50.79%) was comparable to that of right side (51.20%) ($p > .05$). Interestingly, upper third molars and lower third molars had the highest missing rate (97.22% and 83.33%), but there was no statistic difference between them ($p > .05$). Apart from the third molars, the teeth with significantly higher missing rate were upper lateral incisors (83.33%) and upper second premolars (69.44%). The teeth with significantly lower missing rate were upper first molars (33.33%), lower second molars (19.44%), and lower first molars (8.33%). The statistically significant differences of missing rate among tooth positions were presented in Figure 4 ($p < .05$).

Because of the lack of records on deciduous tooth agenesis of *PITX2*-mutated patients in our database and published literatures, the characteristic of deciduous tooth agenesis was not determined. However, as shown in our #535 proband, it seemed that the maxillary deciduous teeth were more susceptible to agenesis than the mandibular ones.

4 | DISCUSSION

As a rare syndrome, ARS can be caused by the mutations in *PITX2* or other genes, but the underlying genetic etiology in approximately 60% ARS cases remains unknown (Seifi & Walter, 2018). In this study, we identified a novel chromosome 4q25 microdeletion including *PITX2*, two novel nonsense mutations (c.148C > T; p.Q50X and c.257G > A; p.W86X), and a frameshift mutation (c.630insCG; p.V211RfsX28) of *PITX2* in four ARS probands. Our data expand the spectrum of *PITX2* mutations and may facilitate the genotype–phenotype analysis in patients with ARS.

Chromosomal microdeletion, a rare type of *PITX2* mutations, is difficult to detect by routine sequencing methods. To date, only 12

TABLE 2 Summary of *PITX2*-related tooth agenesis in permanent dentition

Patients	Diagnose	Mutation		Right quadrants								Left quadrants								Total missing number	Ref
			Max	8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8		
			Mand	8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8		
1	ARS	CNV		■	□	□	□	■	■	■	■	■	■	■	□	□	□	■	■	14	#607
				■	□	□	□	□	■	□	□	□	□	■	□	□	□	□	■		
2	ARS	c.148C>T		■	□	□	□	□	■	□	■	■	□	■	□	□	□	□	■	13	#577
				■	□	□	■	□	■	■	■	■	□	□	□	□	□	□	■		
3	ARS	c.257G>A		■	■	□	■	■	■	■	■	■	■	■	■	■	□	■	■	26	#120
				■	□	□	■	■	■	■	■	■	■	■	■	■	□	□	■		
4	ARS	c.630insCG		■	■	■	■	■	■	■	■	■	■	■	■	■	□	■	■	23	#535
				■	■	□	■	□	□	□	■	■	□	■	□	■	□	□	■		
5	ARS	c.127C>T		■	■	■	□	■	□	□	■	■	□	□	□	■	■	■	■	20	unpublished
				■	■	□	□	■	□	■	■	■	■	□	■	□	□	■	■		
6	ARS	c.216-219delACTT		■	□	■	■	■	■	■	■	■	■	■	■	■	■	■	■	27	Wang et al. 2003
				■	□	□	■	■	■	■	■	■	■	■	■	■	□	□	■		
7	ARS	c.216-219delACTT		■	■	□	■	□	□	■	■	■	■	■	□	■	□	□	■	19	Wang et al. 2003
				■	□	□	□	■	■	■	■	■	■	■	□	□	□	□	■		
8	ARS	c.216-219delACTT		■	■	□	■	□	■	■	■	■	■	■	■	■	■	■	■	24	Wang et al. 2003
				■	□	□	■	□	■	■	■	■	■	■	□	■	□	□	■		
9	ARS	CNV		■	□	■	■	■	■	■	■	■	■	■	■	■	■	□	■	24	Yang et al. 2018
				■	□	□	■	■	■	■	□	□	■	■	■	■	□	□	■		
10	ARS	c.127C>T		■	■	■	■	■	■	■	□	□	■	■	■	■	■	■	■	27	Idress et al. 2006
				■	■	■	■	□	□	■	■	■	■	□	■	■	■	■	■		
11	ARS	c.127C>T		■	■	□	■	□	□	■	■	□	■	□	□	■	□	■	■	20	Idress et al. 2006
				■	■	■	■	□	□	■	■	■	■	□	□	■	□	■	■		
12	ARS	c.127C>T		■	■	■	■	■	□	■	■	■	■	□	■	■	□	■	■	24	Idress et al. 2006
				■	□	□	■	■	■	■	■	□	■	■	■	■	□	□	■		
13	ARS	c.191C>T		■	□	□	□	□	□	■	□	□	■	□	□	□	□	□	■	4	Dressler et al., 2010
				□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□		
14	ARS	c.191C>T		■	□	□	■	□	□	■	□	□	■	■	□	■	□	□	□	8	Dressler et al., 2010
				□	□	□	■	□	□	□	□	□	□	□	□	■	□	□	□		
15	ARS	c.191C>T		■	□	□	□	□	□	□	□	□	□	□	□	□	□	□	■	3	Dressler et al., 2010
				□	□	□	□	□	□	□	□	□	□	□	□	■	□	□	□		
16	ARS	c.205C>T		■	■	□	■	□	□	■	□	□	■	□	□	■	□	■	■	11	Kimura et al., 2014
				■	□	□	□	□	□	□	□	□	□	□	□	■	□	□	■		
17	Non-syndrmoic	c.573-574delCA		■	□	□	■	□	■	■	□	□	■	■	■	■	□	□	■	21	Intarak et al., 2018
				■	□	□	■	■	■	■	■	■	■	■	■	■	□	□	■		
18	Non-syndrmoic	c.573-574delCA		■	■	□	□	□	■	■	□	□	■	■	□	□	■	□	■	14	Intarak et al., 2018
				■	□	□	□	□	■	■	□	□	■	■	□	□	□	□	■		

Note: Data collected from our database (patient 1–8) and literatures (patient 9–18).
Missing teeth are marked with black blocks.

chromosome segmental deletions have been reported in patients with ARS (Yang et al., 2018). In this study, we performed both WES and CNV array for 4 patients with ARS and found a novel de novo 4q25 microdeletion encompassing *PITX2*. The chromosomal microdeletion involving *PITX2* results in haploinsufficiency, which could lead to severe multiple-organ malformations (Idrees et al., 2006; Yang et al., 2018). Among three types of ARS, ARS type 1 patients typically present with ocular and dental abnormalities and most frequently caused by mutations in *PITX2* (Acharya, Huang, Fleisch, Allison, & Walter, 2011; Seifi & Walter, 2018). Indeed, #607 proband carrying a 4q25 microdeletion encompassing *PITX2* presents typical ARS phenotypes, with 14 permanent teeth missing and severe ocular anterior chamber anomalies. However, it is noteworthy that #120 proband with nonsense mutation c.257G > A and #535 proband with frameshift mutation c.630insCG have much more missing permanent teeth than which of #607 proband. Furthermore, #120 proband lost eyesight many years ago, suggesting that she might have the most severe ocular defect among our probands, rather than #607 proband carrying this 4q25 microdeletion. Our data indicate that the clinical manifestations of ARS are diverse, and suggest that phenotypical severity might not be directly correlated to genotype, or PITX2 truncated protein might play a dominant negative role during tooth and ocular development. Nevertheless, further studies are required to elucidate the pathogenic mechanism of *PITX2*-related ARS.

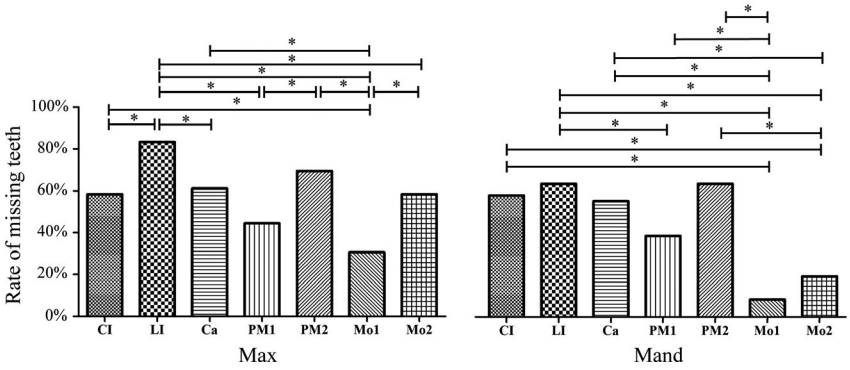
As a downstream target of PITX2 (Green et al., 2001), *DLX2* plays vital roles in regulating the development of branchial arches,

FIGURE 4 The pattern of *PITX2*-associated tooth agenesis. (a) The number of missing teeth in 18 patients with *PITX2* mutations is compiled for each position in the permanent dentition (excluding the third molars) based on our data base and previous reports. The numerators being the number of missing teeth, the denominators being the summation of the teeth that these patients should have at each position. The data for counterpart teeth on the left and right are combined at bottom. The number indicated in brackets denotes the rate of missing teeth. (b) The rate of missing teeth at each maxillary and mandibular tooth position of patients with *PITX2* mutations. The significant difference ($p < .05$) is marked with asterisk. CI: central incisor; LI: lateral incisor; Ca: canine; PM1: first premolar; PM2: second premolar; Mo1: first molar; Mo2: second molar

(a)

Quadrant	Tooth position							Total missing number
	1	2	3	4	5	6	7	
Max R	11/18	15/18	10/18	8/18	12/18	6/18	10/18	72/126
Max L	10/18	15/18	12/18	8/18	13/18	6/18	10/18	74/126
Mand R	11/18	12/18	10/18	7/18	11/18	2/18	4/18	57/126
Mand L	10/18	11/18	10/18	7/18	12/18	1/18	3/18	54/126
Max	21/36 (58.33)	30/36 (83.33)	22/36 (61.11)	16/36 (44.44)	25/36 (69.44)	12/36 (33.33)	20/36 (55.56)	146/252 (57.94)
Mand	21/36 (58.33)	23/36 (63.89)	20/36 (55.56)	14/36 (38.89)	23/36 (63.89)	3/36 (8.33)	7/36 (19.44)	111/252 (44.05)

(b)



and the expression of *DLX2* is required for the tooth and craniofacial development (Qiu et al., 1995; Thomas, Liu, Rubenstein, & Sharpe, 2000). It is suggested that tooth anomaly may be associated with the impaired transcriptional activation capacity of mutant PITX2 on the *DLX2* promoter in patients with ARS (Espinoza et al., 2002). Consistent with this, our luciferase assay results showed that all PITX2 mutants impaired the transcriptional activation of *DLX2* gene, which confirmed the pathogenicity of *PITX2* mutations that we detected in this study. Our data would facilitate the quantitative analysis of the relationship between the pathogenic effects of *PITX2* mutations and the severity of phenotypes in the future.

Previous studies basically divide the clinical manifestations of ARS into ocular and non-ocular systemic defects (Waldron, McNamara, Hewson, & McNamara, 2010), and relatively less attention is paid to deciduous or permanent dental anomalies. In this study, we statistically analyzed the *PITX2*-related tooth agenesis pattern in 18 patients and found that tooth agenesis is more prevalent in the maxilla than in the mandible in permanent dentition, suggesting that development of the maxillary teeth might be more dose-sensitive to PITX2 than which of mandible teeth. The missing maxillary teeth, especially in the upper anterior region, together with the maxillary hypoplasia, would exacerbate the flattened midface. And from the perspective of oral care, both the *PITX2*-mutated patients and their dentists should pay more attention to the remaining maxillary teeth. Although *PITX2* plays important role in left-right asymmetry during multiple-organ development (Gage et al., 1999), our data show that there was no statistically significant difference in number of missing tooth between left and right sides, suggesting that at least in permanent dentition, the contribution of *PITX2* on left-right dental asymmetry is small. Moreover, the third molar agenesis was quite common among general population, with an average worldwide rate of 22.63%. It has been reported that the environmental disturbance, occurring before the initiating of the third molar, was one vital factor causing the third molar agenesis (Swee et al., 2013). In this study, all the 18 *PITX2*-mutated patients missed at least one third molar congenitally, and 15 patients (83.33%) even missed all their third molars, indicating that development of the third molar might be regulated by *PITX2* as well.

Agenesis of deciduous tooth is more rarely seen than which of permanent tooth (Daugaard-Jensen, Nodal, & Kjaer, 1997; Wong et al., 2018). Only a handful of genes have been associated with deciduous tooth agenesis, such as *EDA* (Gaczkowska et al., 2016), *WNT10A* (Yu et al., 2019), *LRP6* (Ockeloen et al., 2016), and *PITX2* (Vande Perre et al., 2018). To our best knowledge, few cases of *PITX2*-related deciduous tooth agenesis were reported. Vande et al. described agenesis of four upper deciduous incisors in a child with a 4q25 microdeletion (GRCh37/hg19 chr4:110, 843, 057–112, 077, 858) encompassing *PITX2* (Vande Perre et al., 2018). Consistent with this, we found that #607 proband had agenesis of four maxillary deciduous incisors, and #535 proband showed agenesis of nine maxillary deciduous teeth. It seems that the maxillary deciduous teeth are more sensitive to *PITX2* mutations; however, the pattern of deciduous tooth agenesis associated with *PITX2* mutations and the different roles of *PITX2* in primary and permanent tooth development need to be further investigated.

In conclusion, here we delineated the detailed clinical features of four patients with ARS, detected a novel 4q25 microdeletion including *PITX2* and three novel *PITX2* mutations, and then analyzed the pattern of *PITX2*-related tooth agenesis. Our findings broaden the spectrum of *PITX2* mutations and offer new dental evidences for genotype–phenotype correlation in ARS. Furthermore, the combined approach of genetic analysis and systematic phenotyping of tooth agenesis we used in this study can better guide health providers in the diagnosis, treatment, and genetic counseling of patients with ARS.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Zhuangzhuang Fan and Shichen Sun contributed to data acquisition, interpretation and drafted manuscript. Haochen Liu, Miao Yu, Ziyuan Liu, Sing-Wai Wong contributed to data analysis. Yang Liu and Dong Han contributed to conception and design, and critically revised manuscript. Hailan Feng contributed to conception of this study.

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