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A novel nonsense mutation in *PAX9* is associated with sporadic hypodontia

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The most important events during the regulation of tooth development were inductive interactions between the epithelial and mesenchymal tissues. The expression of *Pax9* had been shown to specifically mark the mesenchymal regions at the prospective sites of all teeth prior to any morphological manifestations. Here, we investigated the *PAX9* gene as a candidate gene for hypodontia in five unrelated Chinese patients with tooth agenesis. Direct sequencing and restriction enzyme analysis revealed a novel heterozygous mutation c.480C>G (p.160Tyr>X, Y160X) in a patient who was missing 20 permanent teeth (the third molars excluded) and 6 primary teeth. The mutation was a nonsense mutation, leading to a premature stop codon in exon 2 of *PAX9* gene. PCR analysis of complementary DNA from cultured lymphocytes of the affected individual could not indicate the complete degradation of the mutated transcript. Promoter reporter assays revealed reduced transcriptional activity of the mutated PAX9 protein suggesting that the severe phenotype may result from haploinsufficiency of PAX9. In another patient with 15 missing permanent teeth (the third molars excluded), we found the c.219insG mutation previously reported by Stockton.

Introduction

Hypodontia is a tooth developmental anomaly characterised by congenitally missing teeth. It is common in human and has been reported to occur in 0.3–11.3% of the population even if third molars are excluded (1–3). If more than six permanent teeth are congenitally missing, it is called oligodontia. Hypodontia can occur as a part of a syndrome affecting multiple organ systems or it may present in an isolated manner. The non-syndromic form of hypodontia can be sporadic or familial. Familial tooth agenesis is inherited in an autosomal dominant, autosomal recessive or X-linked manner (4–7).

The morphogenesis of teeth is under strict genetic control. The most important events during the regulation of tooth development are inductive interactions between the epithelial and mesenchymal tissues (8). In the early stage of tooth development, the paired domain transcription factor Pax9 is expressed at the prospective sites of all teeth prior to any morphological manifestations or expression of other known transcription factors and signalling molecules (9). A high level of Pax9 expression is maintained throughout the initiation, bud stage and cap stage, and the expression of Pax9 is down regulated at the bell stage (10). In the process, Pax9 mediates its tooth-specific function through its interaction with other proteins. In *Pax9* (−/−) mice, the development of the tooth organ ceases at the cap stage when compared with wild type and the expression of Msx1 and Bmp4 in the mesenchyme is significantly reduced (10). In view of the important role of PAX9 in tooth development, researchers have chosen *PAX9* (MIM: 167416) as a candidate gene for hypodontia and identified 18 distinct disease-causing mutations (11–23). They range from missense mutations that change just one amino acid in the entire protein to premature stop codons that result in truncation of the protein products. Some researchers analysed the function of the PAX9 mutants *in vitro* and suggested that the tooth agenesis phenotype may result from the haploinsufficiency of PAX9 (22,24,25).

In this study, we analysed the coding sequence of *PAX9* in five unrelated Chinese tooth agenesis patients and assessed several functional properties of a new PAX9 mutant.

Materials and methods

Patients

Five unrelated Chinese patients with the diagnosis of oligodontia were enrolled in the present study. Among them, three patients were familial cases and the rest were sporadic cases. The detailed information was listed in Table I. Informed consent was obtained from all individuals. All the study protocols were approved by the Ethics Committee of Peking University Health Science Center (approval number: IRB00001052-06072).

Mutation analyses of PAX9

Genomic DNA was extracted from the peripheral blood leukocytes or the desquamated cells of the study individuals with the use of the TIANamp Blood DNA mini kit (Tiangen, Beijing, China) and the Biotek DNA mini kit (Biotek, Beijing, China) according to the manufacturers' instructions. The entire coding region of the *PAX9* gene was amplified by polymerase chain reaction (PCR) with combinations of intron- and exon-specific primers (primer sequences are available upon request). In a DNA Engine PTC-200 thermocycler, 35 cycles of PCR were performed with denaturation 94°C for 20 sec, annealing at primer-specific temperature for 30 sec and extension at 72°C for 30 sec. PCR products were purified and then sequenced with an ABI 3730 XL automatic sequencer (ABI, USA). DNA sequences were analysed using the databases of NCBI and the BLASTN program (http://www.ncbi.nlm.nlh.gov/BLAST). Each mutation was confirmed in at least two independent experiments by nucleotide sequencing.

Confirmation of identified mutation

For confirmation of the c.480C>G mutation, the 533 bp *PAX9* exon 2 PCR products from the patient and his parents were digested with BfaI (NEB, Beverly, MA, USA) and analysed by 2% agarose gel electrophoresis. The mutant allele is cut by BfaI into 369 and 164 bp fragments, whereas the normal allele is not cut by BfaI. Using this approach, the mutation was also ruled out in 200 control chromosomes (data not shown).

Analysis of PAX9 complementary DNA

Peripheral blood lymphocytes of the patient and his parents were isolated by centrifugation in a lymphocyte separation medium, cultured in RPMI 1640

Table I. Clinical phenotype of the patients

	Gender	Hereditary history	Number of missing teeth	7 7	6 6	5 5	4 4	3 3	2 2	1 1	1 1	2 2	3 3	4 4	5 5	6 6	7 7
1	Female	No	26	●	●	●	●	●	●	●	●	●	●	●	●		●
				●	●	●	●	●	●	●	●	●	●	●	●		●
2	Female	Yes	23	●		●	●	●	●	●	●	●	●	●	●		●
				●	●	●	●	●			●		●	●	●	●	●
3	Male	No	20	●	●	●	●	●					●	●	●	●	●
				●	●	●			●	●	●	●			●	●	●
4	Female	Yes	15	●	●	●	●		●			●		●	●	●	●
				●	●			●								●	●
5	Female	Yes	7	●				●	●			●			●		●
						●											

(●), congenitally missing tooth.

medium supplemented with L-glutamine and 10% fetal bovine serum (FBS) and stimulated with phytohaemagglutinin (PHA, 100 μg/ml) and IL-2 (200 U/ml). Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was treated for 90 min at 37°C with RNase-free DNase, re-extracted with Trizol and reverse transcribed with the use of M-MLV (Fermentas, Canada). The complementary DNA (cDNA) region including the mutant site of *PAX9* gene were amplified by PCR with the following primers: Forward: 5′-GCAAGATCCTGGCGCGATAC-3′ and reverse: 5′-ACTTGGTCGGTGATGGA-3′

The PCR product was analysed by direct sequencing.

Construction of expression vectors

In order to transiently express green fluorescent fusion protein, full-length and truncated *PAX9* cDNA was amplified from pSHlox-*PAX9* plasmid (a generous gift from Dr Annette NeubÜser, University of Freiburg, Germany) by PCR using primers containing BamHI and HindIII sites, respectively. Then, the resulting open reading frame was subcloned into BamHI/HindIII sites of the pEGFP-N1 vector (a generous gift of Dr Y. H. Gan, Research Laboratory of Oral and Maxillofacial Surgery, Peking University School and Hospital of Stomatology). The constructs were completely sequenced to exclude random mutagenesis.

To generate the full-length wild-type and mutant truncated expression plasmid, we amplified *PAX9* cDNA from pSHlox-*PAX9* plasmid by PCR, using the forward primer with the EcoRI site and the reverse primer with the XboI site, and then subcloned into EcoRI/XboI sites of the empty pCMV-HA vector. The construct was completely sequenced to exclude random mutagenesis.

Cell culture, transient transfection and microscopic examination of cells

The human embryonic kidney (HEK) 293A cells (a generous gift of Dr T. J. Li, Department of Oral Pathology, Peking University School and Hospital of Stomatology) were cultured at 37°C and under a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% FBS (Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin.

For transient transfection, 293A cells were trypsinised, counted and plated onto glass coverslips in a 24-well plate at a cell density of 0.5×10^5 per well. After overnight incubation, 600 ng of plasmid DNA was transfected into cells using 2 μl of Lipofectamine 2000 (Invitrogen) following the manufacturer's instruction. Duplicate slides were seeded for each transfection. Forty-eight hours post transfection, the 293A cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature and washed three times with PBS. Coverslips were mounted with Vectashield (Vector Laboratories) and examined with a Leica TCS SP5 confocal microscopy (Leica Microsystems, Heidelberg, Germany).

Cell culture, transient transfection and MSX1 promoter luciferase assay

The culture protocol of COS7 cells was similar to HEK-293A cell. The day before transfection, the COS7 cells were plated on 24-well plate at a cell density of 0.5×10^5 per well. After overnight incubation, cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. A 1000 ng quantity of effector plasmid (pCMV-constructs, wild-type or mutagenised pCMV-constructs) was co-transfected with 250 ng MSX1-luc reporter plasmid (a generous gift of Dr Y. Chai, Center for Craniofacial Molecular Biology, School of Dentistry University of Southern California, Los Angeles). A 10 ng quantity of pRL-SV40 Renilla luciferase vector (a generous gift of Dr Y. Y. Li, Department of Biochemistry and Molecular Biology, Peking University Health Science Center) was also co-transfected for normalisation of transfection efficiency. The cells were harvested 48 h post transfection. The luciferase activity in cell lysates was measured by means of a Dual-Luciferase Reporter Assay kit (Promega, Madison, WI, USA) and a Sirius Single Tube Luminometer (Berthold, Pforzheim, Germany). All transfection experiments were performed in triplicate and repeated at least three times.

Statistical analysis

The results are expressed as means ± SDs of triplicate independent experiments. The data were analysed by Student's *t*-test. P values <0.05 were considered statistically significant.

Result

Mutation analysis and phenotypic correlations

To identify mutations in *PAX9* gene in tooth agenesis patients, we analysed the coding region of the *PAX9* gene. DNA fragments from exon 1 to exon 4 of *PAX9* were amplified from genomic DNA by PCR and directly sequenced. By this approach, two different heterozygous mutations were identified in two probands. One of the mutations, c.480 C>G, is a novel nonsense mutation (Figure 1), and the other one, c.219insG, had been previously reported by Stockton. No mutation was detected in other three cases.

The patient with c.480C>G mutation was a 5-year-old boy (Table I, Patient 3). He and his parents had no health problems related to hair, nails or sweat glands. The patient had 6 primary molars and 20 permanent teeth missing congenitally (Figure 2), including all of his permanent molars. Additionally, his mandibular right primary central and lateral incisors were fused. Both of his parents had two maxillary first molars extracted because of serious caries but had all the other thirty permanent teeth. In addition, the parents' teeth were tetracycline stained.

Direct sequencing indicated an insertion of a guanine at nucleotide 219 of exon 2 of *PAX9* in another female patient with 15 missing permanent teeth (Table I, Patient 4). The same mutation had been reported by Stockton in a family with autosomal dominant oligodontia. It was reported by the mother that the paternal grandfather, father and uncle of the girl were also hypodontia patients (Figure 2b).

Restriction analysis

The novel c.480C>G mutation introduced a new restriction enzyme site: CTAG (BfaI). To confirm this mutation, the 533 bp *PAX9* exon 2 PCR products from the patient and the control population (200 control chromosomes) were digested with BfaI (New England Biolabs, Beverly, MA, USA). The patient's

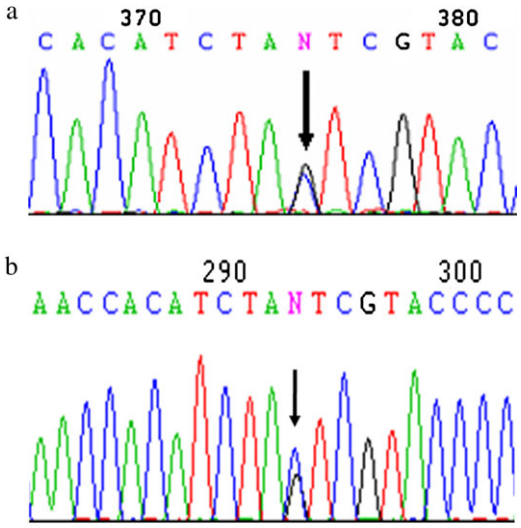

Fig. 1. (**a**) DNA sequencing chromatogram of the *PAX9* gene c.480C>G mutation of the patient. (**b**) DNA-sequencing chromatogram of *PAX9* cDNA amplification products from the patient's lymphocyte cDNA. Arrow indicates the mutant site.

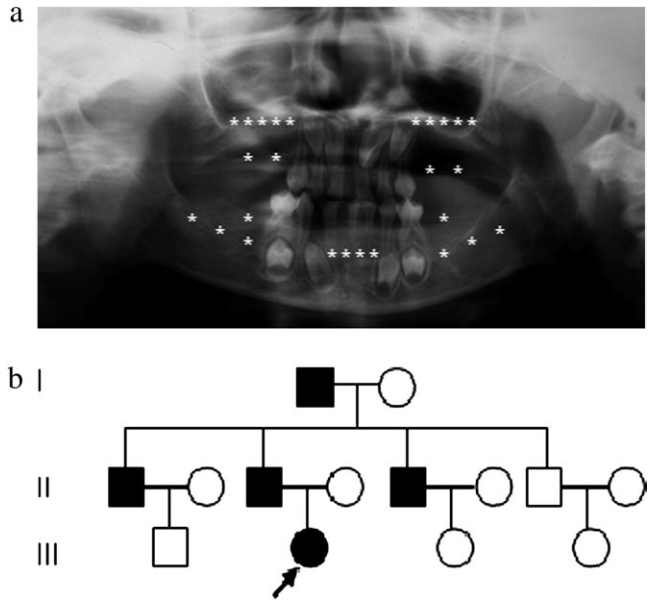

Fig. 2. (**a**) Panoramic radiograph of the c.480C>G mutation patient. (*), congenitally missing tooth. (**b**) Pedigree diagram of the c.219insG mutation family. Arrow indicates the proband.

mutant allele was cut by BfaI into 369 and 164 bp fragments, whereas the normal allele was not cut by BfaI (Figure 3). Sequence analysis and BfaI restriction enzyme digestion of maternal and paternal DNA samples revealed no sequence variation, suggesting that it was a spontaneous mutation.

Analysis of PAX9 cDNA

The amplification product of the patient's lymphocyte cDNA was sequenced directly and showed the mutant nucleotide at the corresponding site indicating that the mutant mRNA was not degraded completely (Figure 1b).

Subcellular localisation of the PAX9 mutants

Nuclear localisation studies by Mensah and Wang (24,26) suggested that abnormal cytoplasmic localisation of PAX9

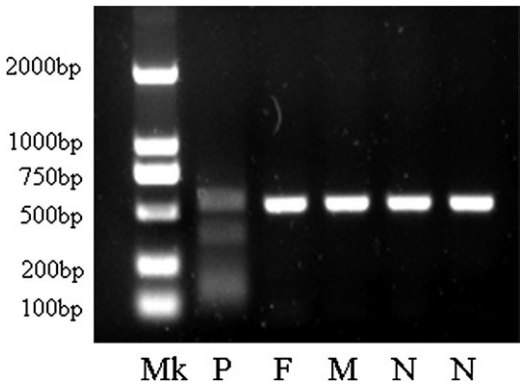

Fig. 3. BfaI restriction enzyme digestion of PCR products of exon 2 of *PAX9*. Mk, DNA marker; P: the patient; F, the father of the patient, M, the mother of the patient; N, normal control. The mutant allele was cut by BfaI into 369 and 164 bp fragments, whereas the wild-type allele was not cut by BfaI.

mutants, in particular the 219insG mutant, could contribute to the loss of function. To test the localisation of Y160X, we constructed wild-type and mutant pEGFPN1-pax9 vector to produce PAX9 fusion protein with a GFP tag at the C-terminus. The constructs were transiently transfected into HEK-293A cells. The mutant PAX9–GFP fusion protein was detected mainly in the nucleus. Its localisation was similar to the wild-type protein (Figure 4).

Msx1-promoter luciferase assay

To evaluate the transactivation activity of the mutant PAX9 proteins, we performed DNA co-transfection experiments in COS7 cells, using the Msx1-promotor-luciferase plasmid as reporter. Msx1 has been well characterised as a direct target gene of PAX9 (22). The result showed that the transcriptional activity of the nonsense mutant was significantly reduced (Figure 5) suggesting that a loss of function as transcription factor is the main cause for the patient's phenotype.

Discussion

In this study, a novel nonsense mutation c.480C>G (p. Tyr160Stop) in *PAX9* was identified in a Chinese sporadic oligodontia patient. This c.480C>G mutation introduces a premature stop codon in the exon 2 of *PAX9* gene and encodes a truncate protein containing the entire paired domain. Frequently, abnormal mRNA with a stop codon in the 5′ part of the coding region will be recognised and removed. Mostowska *et al.* (21) studied the mutation c.619-621delATCins24nt of *PAX9* gene, which causes a premature stop codon in exon 3, and they were not able to demonstrate the presence of the mutant transcript in the patient's leukocytes. To test if the c.480C>G mutation in *PAX9* may target the mRNA to the nonsense-mediated mRNA decay (NMD) process (27), we studied mRNA collected from the patient's lymphocytes by reverse transcription–PCR and sequencing and found that the abnormal mRNA was not completely degraded.

Next, we focused on functional studies of the mutant PAX9 to explain the oligodontia phenotype. The study of Mensah (24) indicated that the 219insG PAX9 mutant could not function properly because it stayed in the cytoplasm, while the wild-type PAX9 was translocated to the nucleus. Cytoplasmatic localisation was not found in eight other tooth agenesis causing Pax9 mutations in the study of Wang *et al.* (26). Our

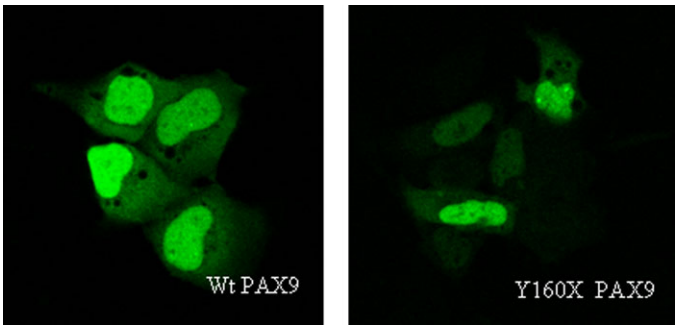

Fig. 4. Subcellular localisation of wild-type and mutant PAX9–GFP fusion protein in HEK-293A cells. The localisation of mutant protein was similar with the wild-type protein's localisation.

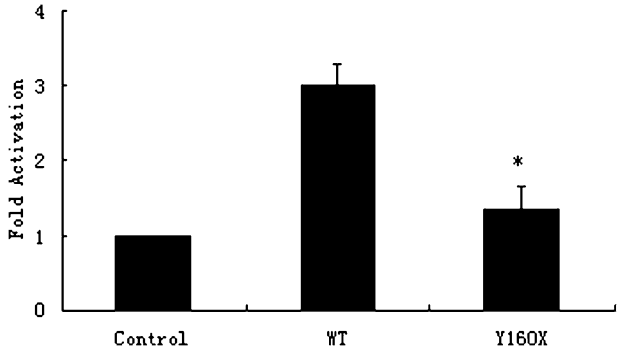

Fig. 5. Transcriptional effects of the wild-type and mutant PAX9 on msx1 promoter in COS7 cells. Values are mean ± SD of three independent experiments performed in triplicate. Error bars represent the standard deviations of the mean; $*P < 0.05$.

result indicated that the Tyr160Stop mutation did not affect the localisation of the PAX9 protein since it was mainly found in the nucleus. However, our reporter gene assay showed that the transactivation ability of the Tyr160Stop mutant was impaired significantly, probably because the nonsense mutation led to the loss of a C-terminal transactivation domain. Our patient misses not only 20 permanent teeth (the third molars excluded) but also 6 primary teeth. The phenotype is similar to the case caused by the deletion of the entire *PAX9* gene reported by Das *et al*. (14). The results of our investigation, similar to previously reported functional studies of *PAX9* mutations, suggest that haploinsufficiency of the *PAX9* gene is probably the cause of this patient's oligodontia. The combined activities of the wild type and mutant alleles could not reach the threshold level necessary for normal tooth development. In this case, the mutation is a *de novo* mutation. The *PAX9* genes of the patient's parents were normal.

Many studies performed in mice suggest that spatially and molecularly distinct signalling pathways create special patterns of dentition (28–30). The current literature on hypodontia suggests that different genes are involved in different types of tooth agenesis (31,32). For example, Kim *et al*. (33) analysed patterns of hypodontia caused by *MSX1* and *PAX9* mutations and found that the most prominent feature of MSX1-associated oligodontia was the frequent absence of maxillary first premolars, while the feature of PAX9-associated oligodontia was the frequent absence of maxillary and mandibular second molars. If we could correlate different clinical phenotypes with different gene mutations clearly, genetic counselling for hypodontia patients would be easier. In this study, we found a previously reported 219insG mutation of *PAX9* in a female patient with 15 missing permanent teeth. She had none of her permanent molars in accordance with the observation that *PAX9* mutations are specifically associated with the agenesis of molars. However, we could not find *PAX9* mutations in the other three patients with missing molars. This may result from mutations in the non-coding regulatory regions of the *PAX9* gene or other tooth agenesis-related genes, and this remains to be clarified in the future.

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Conflict of interest statement: None declared.

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