A novel nonsense mutation in PAX9 is associated with sporadic hypodontia

Junxia Zhu, Xiang Yang, Chenying Zhang¹, Lihong Ge and Shuguo Zheng¹,*

Department of Pediatric Dentistry and ¹Department of Preventive Dentistry, Peking University School and Hospital of Stomatology, 22 Zhongguancun Nandajie, Haidian District, Beijing 100081, China.

*To whom correspondence should be addressed. Department of Preventive Dentistry, Peking University School and Hospital of Stomatology, 22 Zhongguancun Nandajie, Haidian District, Beijing 100081, China. Tel: +86 10 82195510; Fax: +86 10 62173404; Email: zhengsg86@gmail.com

Received on June 15, 2011; revised on September 11, 2011; accepted on September 20, 2011

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 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: IRB000001052-06072).

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.nih.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...
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′-GCAAAGATCTGGCGCGGATAC-3′ and reverse: 5′-AATGGTGCGGTAGTGGA-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. Y. Li, Department of Biochemistry and 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 (BfII). 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 BfII (New England Biolabs, Beverly, MA, USA). The patient’s...
A 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 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-promoter-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. Cytoplasmic localisation was not found in eight other tooth agenesis causing Pax9 mutations in the study of Wang et al. (26). Our

![Fig. 3. BfaI restriction enzyme digestion of PCR products of exon 2 of PAX9.](http://mutage.oxfordjournals.org/)

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.
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.

**Funding**

Beijing Municipal Natural Science Foundation, People’s Republic of China (7022022, 7092112); National Natural Science Foundation, People’s Republic of China (81070815); Youth Foundation of 985 Plan of Medical Science Center, Peking University.

**Acknowledgements**

We are grateful to all the patients and their family members for their participation and contributions. We are grateful to Dr H. X. Meng and Dr Z. B. Chen (Department of Periodontology, Peking University Hospital and School of Stomatology) and Dr A. Mostowska (Department of Biochemistry and Molecular Biology, University of Medical Sciences, Poland) for their technical assistance and guidance.

**Conflict of interest statement:** None declared.

**References**


---

**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 msi1 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.


