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Identification and Functional Analysis of Two Novel *PAX9* Mutations

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Key Words
Oligodontia · Tooth agenesis · *PAX9* · Bmp4 · Missense mutation

Abstract
The paired-domain transcription factor *PAX9* plays a critical role in tooth development, as heterozygous mutations in *PAX9* have been shown to be associated with human tooth agenesis. In this study, we report 2 novel missense mutations, gly6arg (G6R) and ser43lys (S43K), in the paired domain of *PAX9* in Chinese patients with varying degrees of nonsyndromic tooth agenesis. Excluding third molars, the individual with the G6R mutation was missing 2 mandibular incisors and a maxillary premolar, while the phenotype of individuals with the S43K mutation consisted of peg-shaped upper lateral incisors and missing molars, premolars and canines. As these 2 mutations occur at highly conserved amino acids in the PAX gene family and between different species, we further analyzed the effects of the mutations on the function of the resulting proteins. Immunofluorescence and immunoblotting studies showed that the mutations did not alter nuclear localization in mammalian cells. Gel shift and super shift assays indicate that both mutant proteins bound DNA at a lower level than the normal protein, with G6R having a greater affinity for DNA than S43K. Likewise, the G6R protein was able to transcriptionally activate a Bmp4 promoter construct to a greater extent than S43K. Our finding that the severity of tooth agenesis in the patients was correlated to the DNA-binding capacity of the mutated PAX9 9proteins supports the hypothesis that DNA binding is responsible for the genetic defect.

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Introduction

Tooth development involves a complex series of epithelial and mesenchymal signaling interactions [Thesleff, 2003]. Molecular and genetic studies in mice have revealed that more than 300 genes are involved in the process [Thesleff, 2006]. Among these, transcription factors

Abbreviations used in this paper

DHPLC	denaturing high-performance liquid chromatography
EMSA	electrophoretic mobility shift assay
PCR	polymerase chain reaction

Y.W. and H.W. contributed equally to this paper.

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play a prominent role, as evidenced by mutations that are frequently related to human tooth agenesis. Nonsyndromic tooth agenesis can be either sporadic or familial in nature. To date, heterozygous mutations of 2 transcription factors, PAX9 and MSX1, have been most commonly associated with this form of tooth agenesis [Kapadia et al., 2007]. While *MSX1* mutations have been reported to involve cleft lip and palate [van den Boogaard et al., 2000] and Witkop syndrome [Jumlongras et al., 2001], along with missing teeth, all known *PAX9* mutations are associated with nonsyndromic oligodontia that can involve all types of permanent teeth, especially molars. Collectively, these data suggest that *PAX9* plays a dominant role in the development of posterior teeth [Stockton et al., 2000; Nieminen et al., 2001; Das et al., 2002; Frazier-Bowers et al., 2002; Das et al., 2003; Lammi et al., 2003; Mostowska et al., 2003; Jumlongras et al., 2004; Klein et al., 2005; Zhao et al., 2005; Kapadia et al., 2006; Mostowska et al., 2006; Tallón-Walton et al., 2007].

Studies in mice with a homozygous deletion of *PAX9* demonstrate that it has a fundamental role during development [Peters et al., 1998]. These mice lack derivatives of the pharyngeal pouch, have craniofacial and limb anomalies, and fail to form teeth beyond the bud stage of development. Human *PAX9* mutations afford a unique opportunity to investigate how these alterations change gene function and result in the tooth phenotype. Since the initial discovery of a tooth agenesis-causing mutation in *PAX9* [Stockton et al., 2000], a spectrum of autosomal dominant mutations have been identified throughout the entire gene. The majority of mutations is located in the paired domain, the DNA-binding domain of *PAX9* [Kapadia et al., 2007]. As for the functional effect of the mutations, one could predict that the mutant proteins, especially those resulting from a frameshift or nonsense mutation, may result in total loss of function [Stockton et al., 2000; Das et al., 2002, 2003; Klein et al., 2005; Mostowska 2006; Tallón-Walton 2007]. This would imply that haploinsufficiency could be the cause of tooth agenesis. Recent studies of the mutant proteins showed that the loss of DNA binding may explain changes in function [Kapadia et al., 2006; Ogawa et al., 2006]. However, the precise mechanisms for the development of tooth agenesis remain unclear.

In this study, we report the identification of 2 novel missense mutations in the paired domain of *PAX9* in Chinese patients with nonsyndromic tooth agenesis. Based on our functional analysis of the mutant proteins, we propose that the severity of the tooth agenesis phenotypes correlates with the level of functional defects, specifically DNA binding, observed for the respective mutant proteins. This is suggestive of distinct genotype-phenotype correlations for *PAX9* mutations.

Materials and Methods

Subjects

Fourteen unrelated individuals with selective tooth agenesis who showed no signs of other congenital abnormalities or systemic diseases were recruited from the Department of Prosthodontics, School of Stomatology, Peking University. The inclusion criterion was congenital agenesis of at least 1 permanent tooth, not including third molars, as verified by panoramic radiographs and dental history. The family members of all patients were clinically examined and 4 of 14 had 1 family member each who was also affected. In addition, a questionnaire was given to each individual to gather a medical and family history. Seventy individuals with normal number and shape of teeth were recruited as controls. The present study was approved by the Ethics Committee of the Peking University Health Science Center. Informed consent was obtained from all participants, including patients and normal controls.

Mutational Analysis

Peripheral blood samples were obtained for all patients and family members. Buccal swabs were taken from the 70 normal controls. Genomic DNA was isolated and all 4 exons of PAX9 were amplified by polymerase chain reaction (PCR) as previously described [Nieminen et al., 2001]. Denaturing high-performance liquid chromatography (DHPLC) was then performed using the WAVEs DNA Fragment Analysis System (Transgenomic Inc., Beijing, China). The PCR products, which showed abnormal peaks in DHPLC, were gel purified and sequenced as previously described [Wang et al., 2003].

Cell Culture and Transfection

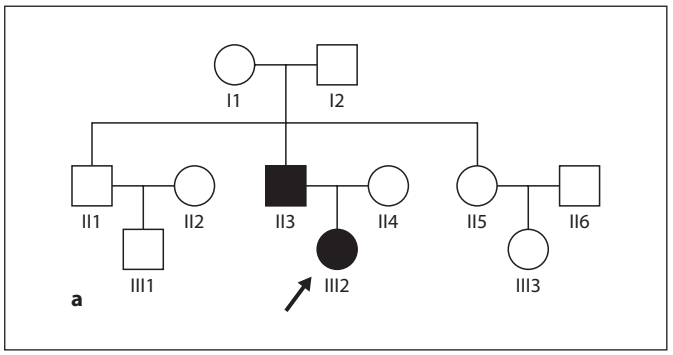
COS7 cells were grown in DMEM (Invitrogen Corp., Carlsbad, Calif., USA) supplemented with 10% fetal bovine serum and maintained at 37°C in the presence of 5% CO_2. They were transfected with plasmids using FuGENE 6 (Roche Diagnostics Corp., Indianapolis, Ind., USA) according to the manufacturer's instructions.

Construction of Expression Plasmids and Site-Directed Mutagenesis

The mammalian expression vector pCMV-Pax9 with c-Myc epitope tag was used as previously described [Kapadia et al., 2006]. To construct pCMV-G6RPax9 and pCMV-S43KPax9, in vitro site-directed mutagenesis was performed using the QuikChange mutagenesis kit (Stratagene Corp., La Jolla, Calif., USA). The mutated constructs were sequenced entirely to confirm the point mutation.

Subcellular Localization

To demonstrate in vivo expression of wild-type and mutant Pax9, COS7 cells were transfected with wild-type Pax9, G6RPax9 or S43KPax9 in the pCMV-Myc vector. Immunolocalization was performed with c-Myc antibody (Santa Cruz Biotechnology Inc.,



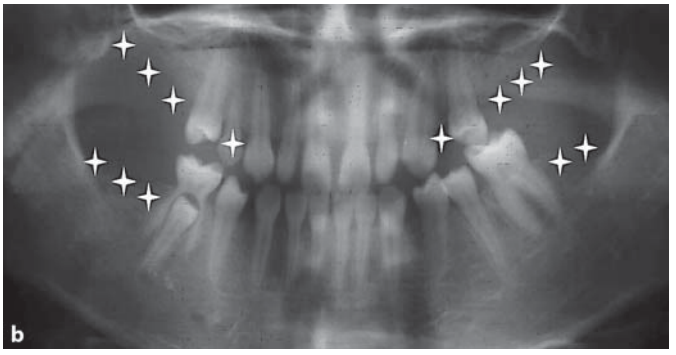


Fig. 1. a Pedigree (circles indicate females, squares indicate males) showing affectation status. The arrow indicates the proband. The pedigree displays an autosomal dominant mode of inheritance. **b** Panoramic radiograph of the proband (III2) at 11 years of age. Stars indicate congenitally missing teeth.

Table 1. Phenotypes of affected family members (III2, II3) and a sporadic case

Mutation	Case	Age		Right								Left							
				8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8
S43K	III2	11	Max	*	*	*		*		p			p		*		*	*	*
			Man	*	*	*												*	*
S43K	II3	38	Max	*					*	p			p	*					*
			Man	*	*													*	*
G6R	sporadic	22	Max	*												*			*
			Man	*							*	*							*

Max = Maxillary; Man = mandibular; * = congenitally missing tooth; p = peg-shaped tooth.

Santa Cruz, Calif., USA) as described previously [Mensah et al., 2004]. Cells were examined 24 h after transfection with a Leica confocal microscope. To confirm our immunocytochemistry results, nuclear and cytoplasmic fractions were obtained from cells transfected for 24 h using the Nuclear Extract Kit (Active Motif, Carlsbad, Calif., USA) according to the manufacturer's instructions. Western blotting was then performed using a previously established protocol [Mensah et al., 2004].

Gel Shift and Super Shift Assay

The oligonucleotide probe used for the gel shift assay was a previously established high-affinity paired domain-binding site, CD19-2(A-ins). The probe was synthesized (Sigma/Genosys, The Woodlands, Tex., USA) and gel retardation assays were performed using a LightShift Chemiluminescent EMSA Kit (Pierce Biotechnology Inc., Rockford, Ill., USA). Biotin-labeled CD19-2(A-ins) was incubated with 1 μg of nuclear extracts from COS7 cells transfected with the appropriate expression plasmid (wild-type Pax9, G6RPax9 or S43KPax9 in pCMV-Myc). After incubation at room temperature for 30 min, 0.2 μg of anti-c-Myc antibody (Santa Cruz Biotechnology Inc.) was added to the reaction mixture and incubated for another 5 min. The entire reaction was loaded onto a 6% nondenaturing polyacrylamide gel. Electrophoretic mobility shift assay (EMSA) was performed in triplicate according to the manufacturer's instructions.

Reporter Assay

PAX9 expression vectors were cotransfected with a Bmp4 promoter-reporter construct (p2.4Bmp4-Luc), described previously [Ogawa et al., 2006]. pCMV-SPORT plasmid (Invitrogen Corp.) was used as the internal control. Cell extracts were prepared with Cell Culture Lysis Reagent (Promega Corp., Madison, Wisc., USA) 24 h after transfection and assayed by luciferase assay system (Promega Corp.) and β-gal assay kit (Invitrogen Corp.).

Results

Identification of Two Novel Missense Mutations in Patients with Varying Degrees of Hypodontia

Two novel missense mutations in the paired domain of *PAX9* were identified in this group of Chinese patients

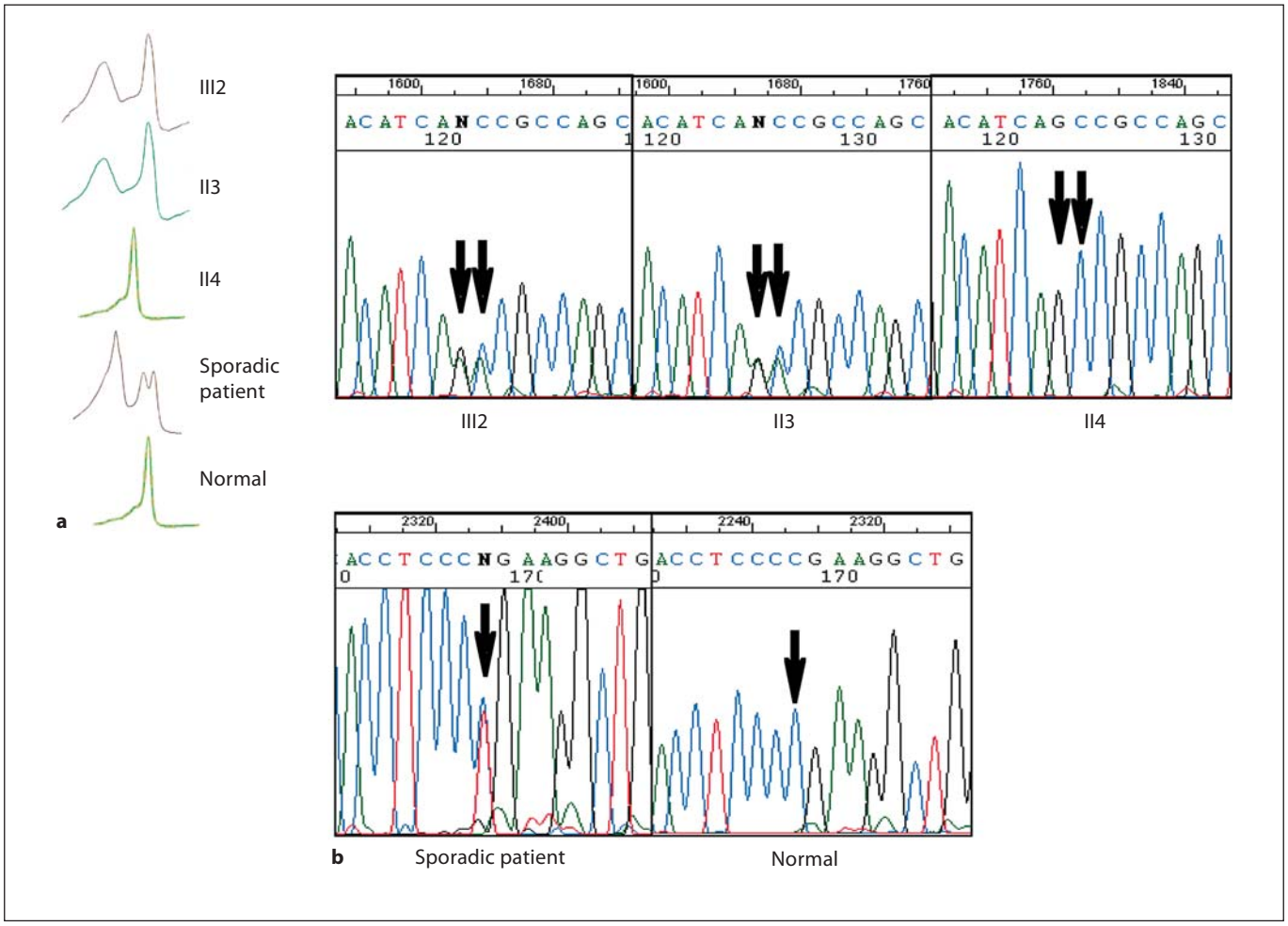


Fig. 2. DHPLC analysis (**a**) and sequence analysis (**b**) of PCR products of *PAX9* exon 2. DHPLC analysis showed abnormal results in the 2 patients of one family and 1 sporadic patient. Direct sequencing of the PCR products accordingly showed the point mutations in exon 2 of the *PAX9* gene (indicated by arrows).

with different levels of hypodontia. The primary dentition was unaffected in all cases. Figure 1 and table 1 show the phenotypes of 3 affected individuals, 2 of whom are related. DHPLC analysis showed abnormal results in the 2 patients of one family and in 1 sporadic patient (fig. 2a). Sequencing analysis revealed 2 different mutations in exon 2 of *PAX9* (fig. 2b). The 2 related individuals carried a double nucleotide mutation, G128A and C129A, which results in the substitution of serine by lysine (S43K). In the sporadic case, a novel heterozygous missense mutation, G16A, would cause a change from glycine to arginine at amino acid 6 (G6R). The pattern of tooth agenesis was significantly different between the 2 cases (table 1). While the individuals in the familial case (S43K) were missing most molars, the sporadic case (G6R) presented with missing mandibular central incisors and a maxillary second premolar. With the exception of the third molars, the molars were not affected. No mutations in *PAX9* were detected in the remainder of the patients and the 26 unaffected relatives from the families. In addition, all 70 control individuals tested lacked these 2 mutations.

Affected Residues Are Evolutionarily Conserved across PAX9 Orthologs and Related Paralogs

Sequence alignment with other members of the *PAX* gene family, as well as the Pax9 gene in other organisms revealed 100% conservation of the 2 affected residues in the paired domain (fig. 3).

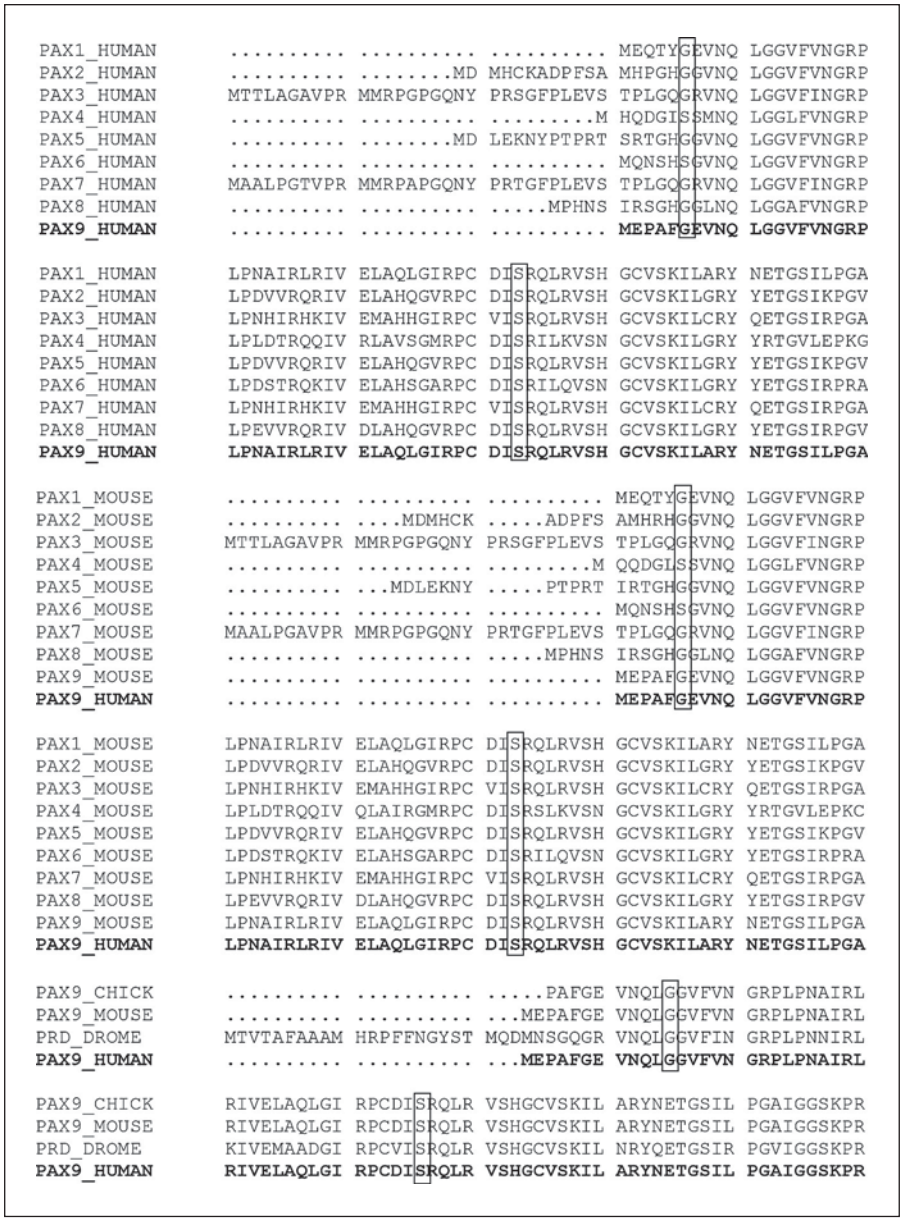


Fig. 3. The mutations cause changes in amino acids, one from glycine to arginine (G6R) and the other from serine to lysine (S43K). Compared with the paired-domain sequence of PAX family genes and between different species, both are evolutionarily conserved.

G6R and S43K Do Not Change Nuclear Localization of PAX9

COS7 cells transfected with an expression vector encoding either wild-type or mutant Myc-tagged *PAX9* showed predominantly nuclear expression by immunofluorescence 24 h after transfection (fig. 4a). Western blot analysis of nuclear and cytoplasmic fractions of COS7 cells 24 h following transfection showed similar results (fig. 4a), confirming that these 2 mutations did not affect nuclear localization of *PAX9*.

G6R and S43K Mutations Cause Decrease in Affinity for DNA

As a transcription factor, PAX9 is likely able to regulate its effector genes by binding to promoter sequences via the paired domain. CD19-2(A-ins), an established paired domain-binding sequence, was chosen to test if the 2 novel mutations affect the DNA-binding ability of PAX9. First, Western blots were performed from nuclear extracts of COS7 transfected with wild-type or mutant *PAX9* expression vector to verify equal expression levels of the proteins. Our EMSA results revealed that both

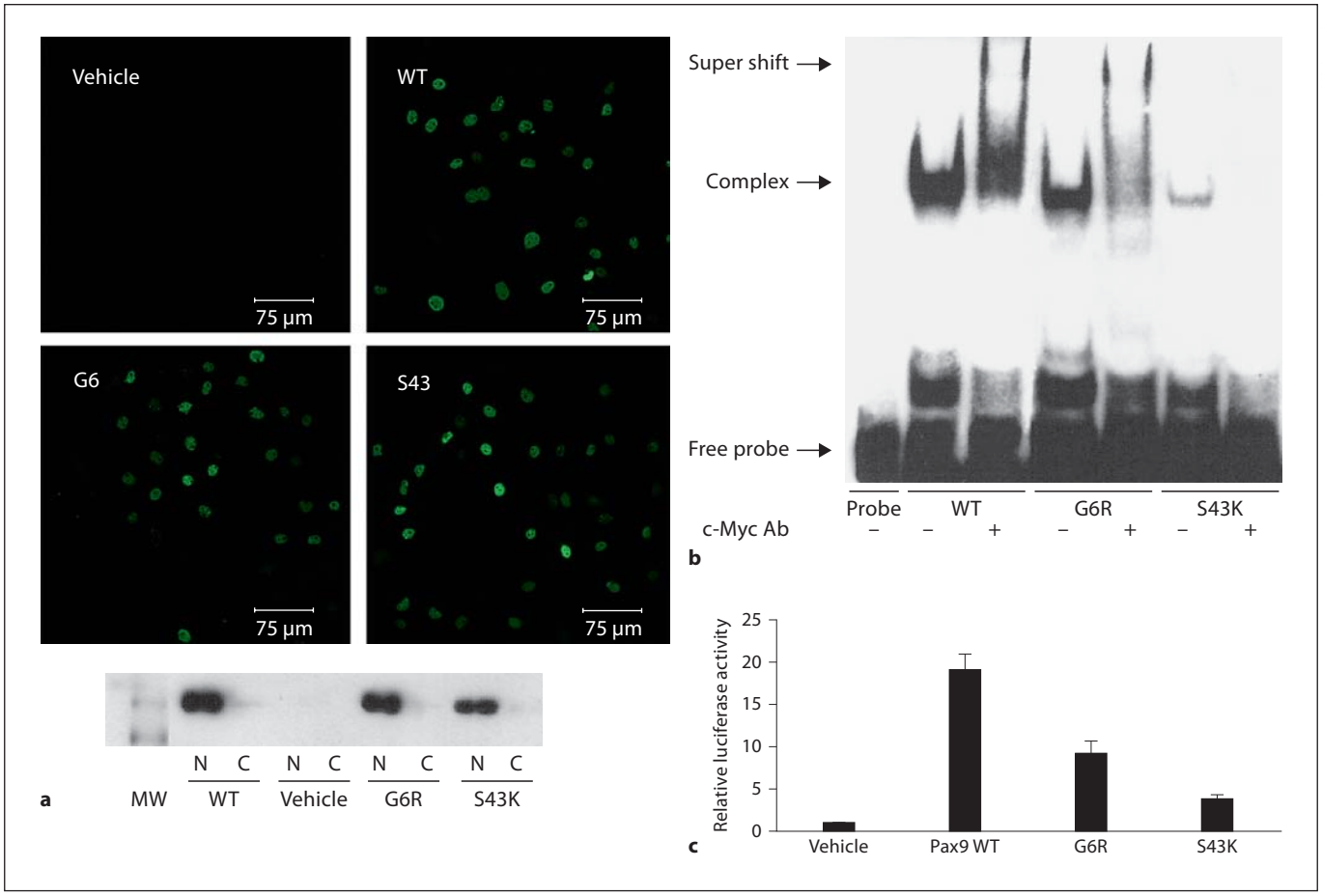


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Fig. 4. a Subcellular localization of G6R and S43K mutant protein does not show any differences when compared to normal protein by immunofluorescence and immunoblotting. **b** Gel shift and super shift assay of nuclear extracts from *PAX9* overexpression COS7 cells shows that like wild-type *PAX9*, both G6R and S43K mutant proteins could form DNA-protein complexes. However, G6R has a higher affinity for DNA than S43K. **c** Luciferase reporter assay using the Bmp4 promoter shows that both G6R and S43K mutant proteins have diminished transcriptional activity. The G6R mutant, however, is able to transcriptionally activate the Bmp4 promoter at a higher level than S43K. The figure represents the results in triplicate. N = Nucleus; C = cytoplasm; MW = molecular weight marker; WT = wild type; Ab = antibody.

mutant proteins have a reduced ability to bind DNA when compared to the wild-type protein. However, the G6R mutant protein had higher affinity for DNA than S43K (fig. 4b).

G6R and S43K Affect Reporter Gene Expression

To further study the in vivo function of the mutant proteins, a luciferase reporter assay was used to test the ability of the proteins to transcriptionally regulate a downstream effector gene, *Bmp4*. Using a Bmp4 promoter reporter construct, the luciferase reporter assay showed a significant reduction in transcriptional activation for both mutants when compared to the wild-type protein. While there was a 2-fold reduction in transcription activity by the G6R protein, the S43K mutant displayed a nearly 5-fold reduction in activity (fig. 4c).

Discussion

In this report, we describe the identification of 2 novel missense mutations in the *PAX9* gene in Chinese patients with nonsyndromic hypodontia. Both mutations (G6R and S43K) localize to the paired domain of *PAX9* and are predicted to affect highly conserved amino acid residues. The clinical phenotypes of the 2 cases are dif-

ferent in terms of severity: excluding the third molar, the patient carrying the G6R mutation is missing only 2 mandibular incisors and 1 maxillary premolar, while patients from the family with the S43K mutation may be missing a number of teeth, including molars, maxillary premolars and mandibular canines. Our functional analysis showed that, although neither mutation altered nuclear localization, the ability to bind DNA and transcriptionally activate a target gene was dramatically reduced. Interestingly, both EMSA and reporter assays showed that G6R protein retained more DNA-binding and transcriptional activation capability, corresponding with the clinical observation of tooth phenotypes. We propose that this loss of function correlates well with the observed phenotypes.

PAX9 as a candidate gene for nonsyndromic tooth agenesis was first reported nearly 7 years ago [Stockton et al., 2000]. Since this initial discovery, other mutations have been identified which present with different phenotypes. Most involved molars, especially the second molars. In this study, the G16A (G6R) mutation shows a surprisingly mild and atypical phenotype where first and second molars were unaffected. While studies in mice have clearly shown that *PAX9* dosage is critical for tooth morphogenesis and differentiation [Kist et al., 2005], the study of naturally occurring mutations in humans affords a unique opportunity to relate different tooth agenesis phenotypes to gene function.

It should be noted that in 15 of the 18 unrelated patients investigated with typical symptoms of nonsyndromic hypodontia, no mutations were found in the coding regions of *PAX9*. This suggests other genes such as *MSX1*, *AXIN2* or some critical noncoding regions of these genes may have variations responsible for the tooth agenesis. Other than syndromic hypodontia, which usually has a clear genetic basis, nonsyndromic hypodontia has an evident difference between the high incidence rate and the relatively small number of reported causative mutations in *PAX9*, *MSX1* or other genes [Gerits et al., 2006]. Thus, it seems that the effect of genetics on tooth agenesis probably is much more heterogeneous than we had expected.

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