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ORIGINAL ARTICLE

Mutations in the PAX9 gene in sporadic oligodontia

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Structured Abstract

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Objectives – Oligodontia, a congenital lack of six or more teeth, is often associated with mutations in the PAX9 gene; therefore, we searched for mutations in this gene.

Design – In the present work, we sequenced fragments of the PAX9 gene in individuals with sporadic oligodontia. Next, we genotyped some mutations we found in patients with oligodontia and individuals without tooth agenesis.

Setting and Sample Population – DNA sequencing was performed in the material isolated from peripheral blood lymphocytes of six unrelated patients with sporadic, non-syndromic oligodontia. These patients were selected based upon explorative cluster analysis. Genotyping was performed in 38 patients with oligodontia and 100 control individuals.

Material and Methods – Direct sequencing and restriction fragment length polymorphism PCR were employed.

Results – We detected two homozygotic substitutions, IVS2-109G > C and IVS2-54A > G, in intron 2 in three patients. Another homozygotic substitution in intron 2, IVS2-41A > G, was revealed in two patients. Two patients had an IVS3+40G > A homozygotic change in intron 3 and 4 patients displayed a 717C > T transition in exon 4 (silent mutation). One patient had a heterozygotic 718G > C transversion, resulting in a missense Ala240Pro substitution. We detected also several other intronic substitutions. Further genotyping of the IVS2-54A > G, IVS2-109G > C, and IVS2-41A > G mutations suggested that they can display polymorphic changes.

Conclusion – The IVS2-54A > G, IVS2-109G > C, and IVS2-41A > G mutations of the PAX9 gene may represent polymorphism associated with sporadic oligodontia.

Key words: genetic polymorphism; oligodontia; PAX9; RNA splicing; tooth agenesis

Introduction

Tooth agenesis affecting up to 20% of the human population is one of the most common congenital disorders. Oligodontia, the absence of six or more teeth excluding third molars, affects up to 1.1% of population (1). There is a wide variation in the location and number of affected teeth, but most frequently lacking teeth are mandibular second premolars, followed by maxillary lateral incisors and the maxillary second premolars (2). Primary teeth are rarely affected (3). Tooth agenesis can be familial or sporadic either associated with a syndrome or non-syndromic (2).

The pathogenesis of tooth agenesis is largely unknown. The etiology of congenital absence of teeth is believed to be associated with heredity or developmental anomalies, often determined by mutations. There is a discrepancy between the high incidence of agenesis and the relatively small number of reported causative mutations in candidate genes, which suggests that the role of genetic factors in oligodontia is rather complex (4). These genetic factors interact with the environment in an age-dependent manner. The study performed in mice allowed to identify a set of candidate genes for inherited tooth loss, but mutations in only three of them have been identified in human pedigrees with familial hypodontia or oligodontia: PAX9, MSX1, and AXIN2 (5). Mutations of the PAX9 and MSX1 genes disturb regulatory processes of tooth formation (6).

Apart from the PAX9, MSX1, and AXIN2 genes, also other genes may be related to non-syndromic oligodontia. The preferential premolar agenesis can be associated with the IRF6 (interferon regulatory factor 6) gene, which interacts with MSX1 and tumor growth factor alpha as well as fibroblast growth factor receptor 1 (7). Additionally, dental agenesis can be related to ectodermal dysplasia condition, which depends on many factors, like the diet or a disease resulting from a bacterial infection (8, 9). Another gene associated with syndromic hypodontia, ectodysplasin A (EDA), is also involved in ectodermal dysplasia. The product of the EDA gene is a type-II transmembrane protein-containing tumor necrosis factor homology domain, and the missense mutations, Q358E, D316 G, and T338M, in this domain results in hypodontia phenotype (10, 11). The EDA gene is still the subject of study; and novel missense mutations, A259E, R289C, and R334H, were identified in non-syndromic oligodontia (12).

The PAX9 gene encodes for transcription factors that function in tooth development. The gene is expressed in dental mesenchyme at all stages of odontogenesis (13). The products of the PAX9 gene facilitate cross-talk between epithelial and mesenchymal tissues and are essential for the establishment of odontogenic potential of the mesenchyme (14). The expression of the PAX9 gene is a marker for tooth formation and occurs before any morphological manifestation of this process takes place (15). PAX9 has four exons, and exon 2 contains a paired box, a sequence encoding a specific DNA-binding domain. Since the initial discovery of an insertion mutation within the paired domain of PAX9, a number of mutations, both missense and nonsense, have been associated with tooth agenesis (1, 16). Known mutations are spread over the entire gene with hot spots in the paired domain and many of them may alter or impair PAX9 functions (17, 18).

In the present work, we searched for mutations in the PAX9 gene in six unrelated individuals affected by sporadic, non-syndromic oligodontia. Moreover, we checked the presence of these mutations in cohorts of subjects with oligodontia and individuals without tooth agenesis to determine whether some of them may be polymorphisms.

Materials and methods

Patients

Six unrelated patients with sporadic, non-syndromic oligodontia were enrolled in this study for sequencing the PAX9 gene. They were selected based on explorative cluster analysis, as described elsewhere (19). The analysis assigned 44 patients with sporadic, non-syndromic oligodontia in the PAX9 cluster, with six individuals grouped in the close vicinity of the center of gravity of this cluster. DNA fragments containing the PAX9 gene from these patients were sequenced. They all were minors. Furthermore, 32 patients with non-syndromic oligodontia were enrolled for PCR genotyping of the mutations detected by sequencing. A hundred individuals without tooth agenesis were enrolled as controls and genotyped in the same way. They all were patients of the Department of Pediatric Dentistry, Medical University of Lodz, Lodz, Poland. The participants signed the informed consent form along with their parents, if necessary. The protocol of the study was reviewed and approved by the Ethic Committee of the Medical University of Lodz, Poland. Clinical inspection of parents' teeth was performed and a dental synopsis was obtained, followed by collecting a peripheral blood sample and taking a panoramic radiograph from each patient (Fig. 1).

To the best of our knowledge obtained by the clinical check-up or/and dental history, the parents' examination did not reveal any congenital lack of teeth excluding third molars. Oculo-digito-cutaneous signs of abnormality were excluded both in the proband and in the respective parents. Dental history of each patient was surveyed.

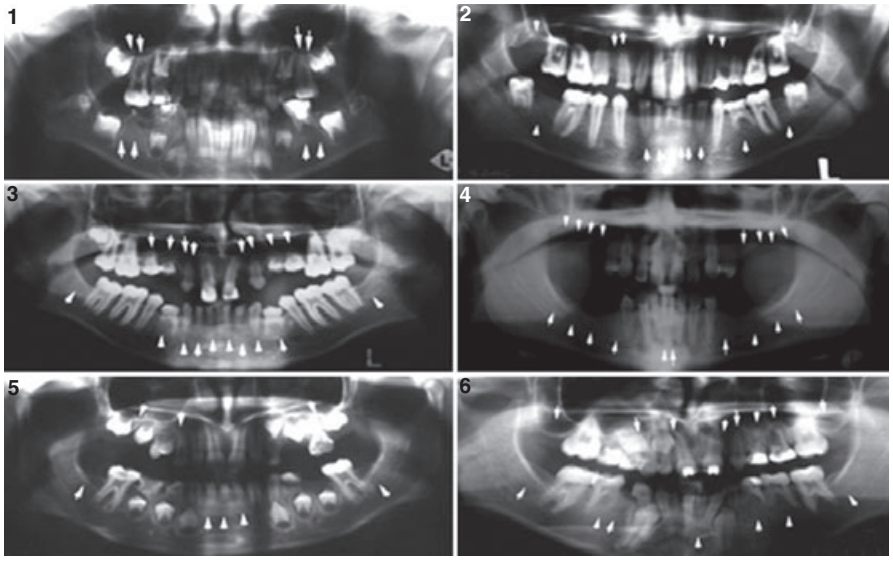

Fig. 1. Panoramic radiograph of the dentition. Arrows indicate lacking teeth. The numbering of the diagrams corresponds to the numbering of the patients given in Table 2.

PAX9 sequencing

DNA was isolated from the peripheral blood of the patients in a MagNA Pure Compact Instrument robotic station with MagNA Pure Nucleic Acid Isolation Kit (Roche, Rotkreuz, Switzerland). Four contigs covering all four exons, exon-intron boundaries and 5′- and 3′-UTRs of PAX9 with their surrounds were chosen for gene sequencing. The contig 1 was located from −187 to +270 (all locations in bp from the transcription start site), covering promoter, exon 2 and 5′-part of the intron 2, contig 2 spanning +906 to +1770 and covering exon 3 and boundary sequences of the intron 3, contig 3 (+4496 to +4831) covering exon 4 and boundary sequences of intron 4, and contig 4 (+11 369 to +15 990) covering part of the + 3′UTR.

DNA was amplified by polymerase chain reaction (PCR) with the following primers (all sequences in 5′ → 3′ direction): contig 1 - forward (F): TCTTTTTGT GCCGCTTCTGG, reverse (R): GCTCCCTCCCTCCCTTCC; contig 2 (sequence of nested primers in parentheses): F: TCGGCTATGTTCAGGGACC (CGGACCTACAAGCAG AGAGA), R: AGGTGGTGGGAAAGACAGTG (GGTGCTGC TTGTATGAGTCG); contig 3: F: TGTAAAACGACGGC-CAGTAGGGAGCCGACCCAAGGTCT, R: CAGGAAACAG CTATGACCGCGGGAAGCTCAGAAAGGGA; contig 4: F: CATTGCTGGCTTACTCAGACTTA, R: TGCTCACAC-ACACATATACTCCAC. The PCR was carried out in a MJ Research thermocycler, INC thermal cycler, model PTC-225 (Waltham, MA, USA). Each 25 μl of the PCR contained (concentration varied depending on the contig): 10 ng genomic DNA, 1.0–1.7 U Taq polymerase (Fermentas, Vilnus, Lithuania or Sigma, St. Louis, MO, USA) in 1 × PCR buffer (100 mM Tris–HCl, pH 8.3, 500 mM KCl, 11 mM $MgCl_2$, 0.1% gelatin), 1.1–2.1 mM $MgCl_2$, 0.30–1.20 mM dNTPs, and 250 nM of each primer. Thermal cycling conditions were as follows: initial denaturation step at 95°C for 3 min, then 50 s at 95°C and 38 cycles for 25 s at the 60.0–60.5°C annealing temperature, and at 72°C for 40 s. The final extension step was performed at 72°C for 7 min. Purified PCR products were directly sequenced in an Applied Biosystems sequencer, model ABI 3730 (Applied Biosystems, Foster City, CA, USA). Representative results of the sequencing are displayed in Fig. 2. Numbering in the sequences was within a corresponding cDNA, in which A in ATG codon is number +1.

Determination of the PAX9 genotype

DNA was isolated from peripheral blood leukocytes by using proteinase K digestion and phenol/chloroform extraction. The IVS2-41A > G polymorphism of the PAX9 gene was typed by allele-specific oligonucleotide PCR (ASO-PCR) (Table 1). The IVS2-54A > G and IVS2-109G > C mutations of the PAX9 gene were determined by the restriction fragment length polymorphism PCR (RFLP-PCR). The PCR was carried out in a PTC-200 DNA Engine® Thermal Cycler (BIORAD, Hercules, CA, USA) in a total volume of 25 μl, containing 10 ng genomic DNA, 1 U MasterAmp™ Taq polymerase (Epicentre, Madison, WI, USA) in 1 × PCR buffer

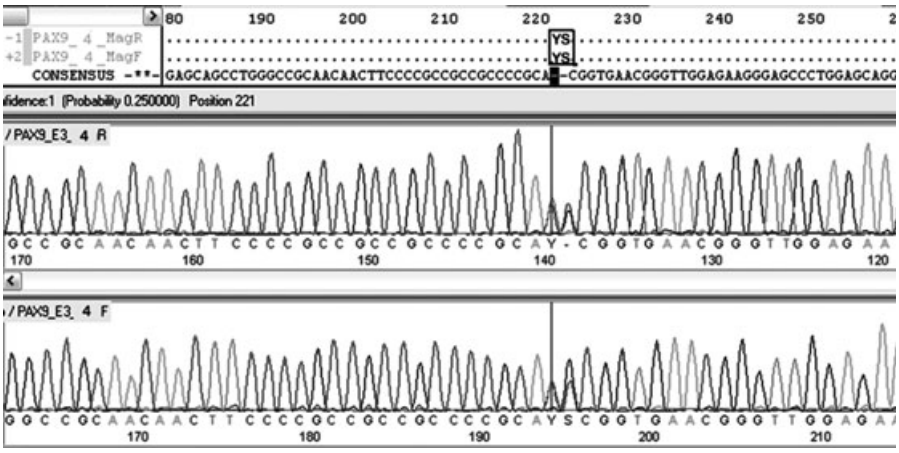

Fig. 2. Representative chromatograms of individuals affected by oligodontia (patient nr 4) showing two heterozygotic substitutions (framed): 717C > T and 718G > C in exon 4.

Table 1. **Genotyping of the PAX9 gene**

Mutation	Primers	Ta	Restriction enzyme	PCR product's length	Restriction fragments	Digested allele
IVS2-41A > G						
Sense A	5′-TGGCAGGCAGCTGTCCCAAGCG-5′	68°C	–	408 bp	–	–
Sense G	5′-TGGCAGGCAGCTGTCCCAAGCA-3′					
Antisense	5′-TTGTACTTGTCGCACACGCCGTCC-3′					
IVS2-54A > G						
Sense	5′-TCGGCTATGTTCAGGGACC-3′	54°C	*Alu*I	163 bp	108. 55 bp	A
Antisense	5′-GCRCTGCAAAATGAACACACAG-3′					
IVS2-109G > C						
Sense	5′-TCGGCTATGTTCAGGGACC-3′	54°C	*Rsa*I	163 bp	113.50 bp	C
Antisense	5′-GCRCTGCAAAATGAACACACAG-3′					

Ta, annealing temperature.

(75 mM Tris–HCl, pH 9.50 mM KCl, 2 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$), 3 mM $MgCl_2$, 0.2 mM dNTPs and 0.4 μM of each primer (POLGEN, Lodz, Poland). The thermal cycling conditions were 5 min at 95°C, followed by 30 cycles of 30 s at 95°C, 45 s at 68°C or 54°C, and 1 min at 72°C. The final extension step was performed at 72°C for 3 min. A 408 bp of ASO-PCR-amplified product was analyzed on a 3% agarose gel and visualized by ethidium bromide staining. Typical results of genotype analysis by allele-specific oligonucleotide PCR are displayed in Fig. 3. A 163-bp RFLP-PCR products were digested separately for 2 h by 2 U (units) of the restriction enzymes: *Alu*I or *Rsa*I (Fermentas, Vilnius, Lithuania). The A allele of the VS2-54A > G mutation was digested by *Alu*I into 108- and 55-bp fragments, whereas the G variant remained intact. In turn, the C allele of IVS2-109G > C mutation was digested by *Rsa*I into 50- and 113-bp fragments, whereas the G variant remained intact. Digested products were separated onto a 12% polyacrylamide gel and visualized by ethidium bromide staining. Typical results of genotype analysis by RFLP-PCR are displayed in Figs 4 and 5.

Statistical analysis

The allelic frequencies were estimated by gene counting, and the genotypes were scored. The χ^2 analysis was used to compare the observed number of genotypes with that expected for a population in the Hardy–Weinberg equilibrium. The χ^2 analysis was also used to test the significance of the differences in the observed alleles and genotypes between the affected and control groups. A logistic regression model was used to calculate the odds ratios (ORs) and 95% confidence intervals (CIs). Analyses were performed with STATISTICA 6.0 software (Statsoft, Tulsa, OK, USA).

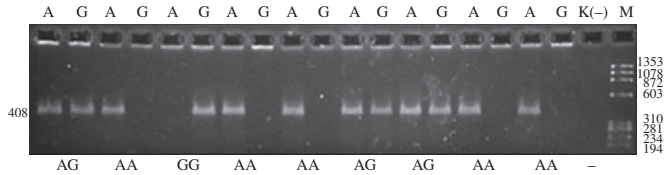

Fig. 3. Genotypes of the IVS2-41A > G polymorphism of the PAX9 gene determined by allele-specific PCR detection and analyzed by 3% agarose gel electrophoresis, stained with ethidium bromide and viewed under UV light. Lane K(–) displays the negative control of PCR reaction (without DNA). Lane M displays ΦX 174/*Bsu*RI molecular weight marker; lanes marked by A and G show the results of amplification with primers specific to the A and G alleles, respectively. Genotypes are indicated in the lower part of the picture.

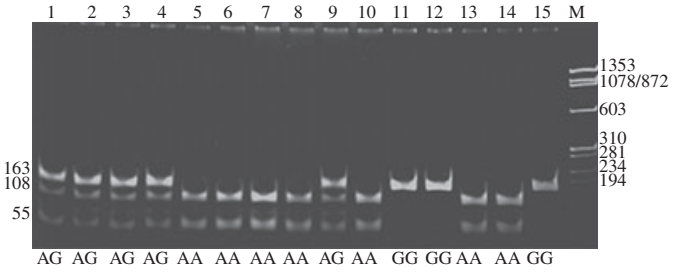

Fig. 4. Representative restriction fragment length polymorphism PCR analysis of the IVS2-54A > G of the PAX9 gene. Lanes 1–4, 9 heterozygotes (AG) contain three bands (163, 108 and 55 bp) following restriction digestion by *Alu*I. Lanes 11, 12, and 15 IVS2-54A > G homozygotes (GG) are not cleaved by *Alu*I enzyme and remain a single 163-bp band. Lanes 5–8, 10, 13, and 14 are cleaved by *Alu*I and yield 108- and 55-bp bans. Lane M, DNA marker ΦX174 DNA/*Bsu*RI. Digested products were electrophoresed on a 12% polyacrylamide gel and visualized by ethidium bromide staining. PAX9 IVS2-54A > G PCR-RFLP band sizes are indicated on the left of the panel.

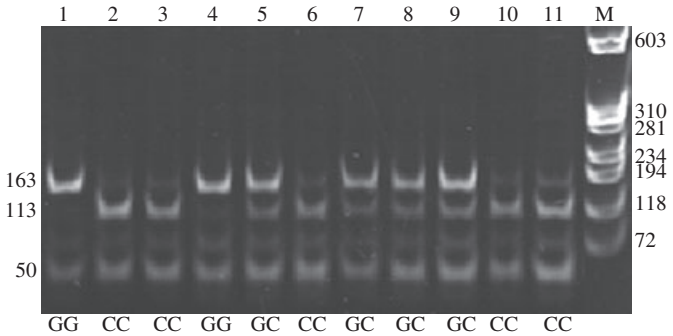

Fig. 5. Representative restriction fragment length polymorphism PCR analysis of the IVS2-109G > C of the PAX9 gene. Lanes 5, 7–9 heterozygotes (GC) contain three bands (163, 113, and 50 bp) following restriction digestion by *Rsa*I. Lanes 1 and 4 IVS2-109G > C homozygotes (GG) are not cleaved by *Rsa*I enzyme and remain a single 163-bp band. Lanes 2, 3, 6, 10, and 11 are cleaved by *Rsa*I and yield 113- and 50-bp bans. Lane M, DNA marker ΦX174 DNA/*Bsu*RI. Digested products were electrophoresed on a 12% polyacrylamide gel and visualized by ethidium bromide staining. PAX9 IVS2-109G > C PCR-RFLP band sizes are indicated on the left of panel.

Results

We detected several mutations in oligodontia-affected patients when compared to the cDNA sequence (Table 2). We observed the presence of two homozygotic substitutions, IVS2-109G > C and IVS2-54A > G, in intron 2 in three patients, number 4, 5, and 6. Another homozygotic substitution in intron 2, IVS2-41A > G, was detected in 2 patients, number 5 and 6. The IVS3+40G > A homozygotic transition in intron 3 was revealed in patients 5 and 6. These patients had the 717C > T transition in exon 4; and additionally, patient 6 had heterozygotic transition, 1579G > C, in 3′-UTR, present also in patient 4, and patient 5 had a heterozygotic substitution 1843C > T, which was also present in patient 1, and patient 3 had its homozygotic T/T variant. Patient 4 was the only one, who had a heterozygotic transition 717G > C in exon 4. This mutation resulted in a missense amino acid substitution Ala240Pro. No mutations were detected in patient 2.

Next, we checked the presence of IVS2-54A > G, IVS2-109G > C, and IVS2-41A > G variants in non-related 38

Table 2. **Mutations (in bold) in the PAX9 gene in patients with oligodontia**

	Location							
	IVS2-109G > C	IVS2-54A > G	IVS2-41A > G	IVS3+40G > A	717C > T	718G > C	1579G > C	1843 C > T
Patient	Intron 2	Intron 2	Intron 2	Intron 3	Exon 4 rs12881240	Exon 4 rs4904210	3′ UTR	3′ UTR
Reference*	G/G	A/A	A/A	G/G	C/C	G/G	G/G	C/C
1	G/G	A/A	A/A	G/G	C/C	G/G	G/G	C/**T**
2	G/G	A/A	A/A	G/G	C/C	G/G	G/G	C/C
3	G/G	A/A	A/A	G/G	C/C	G/G	G/G	**T**/**T**
4	**C**/**C**	**G**/**G**	A/**G**	G/**A**	C/**T**	G/**C**	G/**C**	C/C
5	**C**/**C**	**G**/**G**	**G**/**G**	**A**/**A**	**T**/**T**	G/G	G/G	C/**T**
6	**C**/**C**	**G**/**G**	**G**/**G**	**A**/**A**	**T**/**T**	G/G	G/**C**	C/C

*cDNA +1 **A**TG; Mutations are in bold.

individuals with non-syndromic oligodontia and 100 controls without tooth agenesis. All distributions of genotypes, except that for the IVS2-54A > G polymorphism in control, did not differ from those expected by the Hardy–Weinberg equilibrium. We observed an association (OR 4.86, 95% CI 1.66–11.86) between the occurrence of oligodontia and the A/A genotype of the IVS2-54A > G substitution (Table 3). We did not find any association between the IVS2-109G > C and IVS2-41A > G variants and oligodontia (Table 3). The haplotypes of the IVS2-54A > G/IVS2-109G > C (OR 1.71, 95% CI 1.14–2.58), IVS2-54A > G/IVS2-41A > G (OR 1.98, 95% CI 1.32–2.97), and IVS2-109G > C/ IVS2-41A > G (OR 1.60, 95% CI 1.07–2.38) variants were positively correlated with the oligodontia (Table 4). Distribution of triple haplotypes of all three mutations showed that GGG and AAA increased the risk of oligodontia, whereas the GGA, AGG, and AGA haplotypes decreased it (Table 4).

Discussion

Oligodontia can be categorized into familial and sporadic. Whereas in the familial form suspected causative mutations can be established on the basis of the same genotype in all affected members of the family, determination of mutations associated with sporadic oligodontia is more complex.

At first, the traits in patient 1 reminded us of Kabuki syndrome (20), because macrodontia of the upper central incisors with large notches suggesting tendency to gemination and concomitant oligodontia was observed. Additionally, the boy displayed hyperdontia in the upper anterior primary and lower permanent teeth with general enamel hypoplasia of permanent dentition. Archived dental history confirmed by an x-ray at the age of six showed six mandibular deciduous incisors and five in the maxilla. Taking into account the discovery of Kjaer (21), we decided to accept middle incisors as additional. The boy received consulting in clinical genetics and revealed no facial dysmorphism, neither short stature or electroencephalography (EEG) abnormalities nor any other ecto-mesenchyme-derived disorders. Therefore, we excluded the Kabuki syndrome in patient 1. Oligopleiodontia (simultaneous hypo- and hyperdontia) has been explained as a reaction of epithelium to developmental disturbance (22). Therefore, the same molecular code damage could result in the unbalanced dental phenotype. Although multiple traits in the dentition were confusing, oligodontia along with the position displacement of the second permanent molars prompted us to screen PAX9 mutation (15). However, the dentition pattern of this patient suggests that he has not only an isolated oligodontia, but also macrodontia, hyperdontia, enamel hypoplasia. We speculate that this may be underlined by the reduction in the PAX9 gene dosage in this patient, resulting in the tooth disturbances in the oral proximal tooth-forming regions (23).

The most striking result was the presence of two homozygotic substitutions, IVS2-109G > C and IVS2-54A > G, in three patients. Apparently, an immediate question arises about the functional significance of these mutations. It is well established that the

Table 3. **Distribution of genotypes, frequency of alleles of the IVS2-54A > G, IVS2-109G > C, and IVS2-41A > G mutations in the PAX9 gene and odds ratio (OR) with 95% confidence interval (95% CI) in patients with oligodontia and individuals without tooth agenesis (controls)**

	Patients (n = 38)		Controls (n = 100)		
Genotype or Allele	Number	Frequency	Number	Frequency	OR (95% CI)
IVS2-54A > G					
A/A	9	0.24	6	0.06	**↑4.86 (1.66–11.86)**
A/G	15	0.39	60	0.6	**↓0.43 (0.20–0.93)**
G/G	14	0.37	34	0.34	1.13 (0.52–2.47)
	χ^2 = 1.47		χ^2 = 9.12		
A	33	0.43	72	0.36	1.36 (0.80–2.34)
G	43	0.57	128	0.64	0.73 (0.43–1.25)
IVS2-109G > C					
G/G	9	0.24	13	0.13	2.08 (0.80–5.36)
G/C	15	0.39	59	0.59	**↓0.45 (0.21–0.97)**
C/C	14	0.37	28	0.28	1.50 (0.68–3.31)
	χ2 = 1.46		χ2 = 4.29		
G	33	0.43	85	0.43	1.04 (0.61–1.77)
C	43	0.57	115	0.57	0.96 (0.57–1.64)
IVS2-41A > G					
A/A	15	0.39	29	0.29	1.60 (0.73–3.49)
A/G	17	0.45	57	0.57	0.61 (0.19–1.30)
G/G	6	0.16	14	0.14	1.15 (0.41–3.26)
	χ^2 = 0.10		χ^2 = 2.76		
A	47	0.62	115	0.58	1.20 (0.70–2.06)
G	29	0.38	85	0.43	0.84 (0.49–1.43)

Significant ORs are in bold.

mutations affecting the critical paired domain of the PAX9 gene located in exon 2 may be important for pathogenesis of oligodontia. Intronic sequences can be important for both normal and alternative splicing, which is one of the main mechanisms of the regulation of the human genome expression (24). It is suspected that nearly all protein-encoding genes will be found to utilize alternative splicing, at least to some extent.

The lack of any mutation in the patient number 2 is somehow surprising. However, it should be remembered that we did not analyze the sequence of the entire PAX9 gene. First, the first contig was spanned from the −187 position, but we can expect that the 5′ regulatory region spans far downstream from this position. Second, only the parts of introns positioned next to exons were sequenced and we cannot be sure, whether this patient had any intronic mutation. Another problem is the question about its functional significance.

Several heterozygotic mutations were detected in our research. It was reported that Pax9−/− mice displayed several cranio-facial anomalies, but heterozygous mutants did not exhibit any obvious abnormalities (25). However, the vast majority of all known PAX9 mutations resulting in human agenesis are heterozygous.

The expression and the functional characterization of the mutant gene as well as creation of a transgenic animal bearing these mutations may advance our understanding of their role in oligodontia. We do not have detailed information on sequences in the PAX9 gene, which can be involved in alternative splicing, but we know that not only sequences of the 5′ and 3′ splicing sites, pyrimidine-rich tract, and branch site may play a role in this process. Because canonical splicing sites are short and mostly degenerated, there is an apparent need for additional *cis*-acting signals to guide splicing machinery away from potential but incorrect splicing sites toward proper splicing sites. The

Table 4. **Distribution of haplotypes of the IVS2-54A > G, IVS2-109G > C, and IVS2 -41A > G mutations in the PAX9 gene and odds ratio (OR) with 95% confidence interval (95% CI) in patients with oligodontia and individuals without tooth agenesis (controls)**

	Patients (n = 38)		Controls (n = 100)		
Haplotype	Number	Frequency	Number	Frequency	OR (95% CI)
IVS2-54A > G/IVS2-109G > C					
AG	51	33.55	91	22.75	**↑1.71 (1.14–2.58)**
AC	15	9.87	53	13.25	0.72 (0.39–1.31)
GG	15	9.87	79	19.75	**↓0.44 (0.25–0.80)**
GC	71	46.71	177	44.25	1.10 (0.76–1.61)
IVS2-54A > G/IVS2-41A > G					
AA	56	36.84	91	22.75	**↑1.98 (1.32–2.97)**
AG	10	6.58	47	11.75	0.53 (0.26–1.08)
GA	38	25.00	139	34.75	**↓0.63 (0.41–0.95)**
GG	48	31.58	123	30.75	1.04 (0.69–1.55)
IVS2-109G > C/IVS2-41A > G					
GA	56	36.84	107	26.75	**↑1.60 (1.07–2.38)**
GG	10	6.58	59	14.75	**↓0.41 (0.20–0.82)**
CA	38	25.00	123	30.75	0.75 (0.49–1.15)
CG	48	31.58	111	27.75	1.20 (0.80–1.80)
IVS2 54A > G/IVS2 109G > C/IVS2 -41A > G					
GGG	92	30.26	114	14.25	**↑2.61 (1.91–3.58)**
GGA	10	3.29	68	8.50	**↓0.37 (0.19–0.72)**
GAG	20	6.58	68	8.50	0.76 (0.45–1.27)
GAA	10	3.29	38	4.75	0.68 (0.34–1.39)
AGG	20	6.58	88	11.00	**↓0.57 (0.34–0.94)**
AGA	10	3.29	70	8.75	**↓0.35 (0.18–0.70)**
AAG	56	18.42	178	22.25	0.79 (0.56–1.10)
AAA	86	28.29	176	22.00	**↑1.40 (1.04–1.89)**

Significant ORs are in bold.

best characterized such sequences are enhancers and silencers, which are targets for serine-rich proteins and proteins that bind heterogenous nuclear RNA (hnRNP proteins) (26). The latter could be involved in exon juxtaposition (27). hnRNP proteins can repress splicing by directly antagonizing the recognition of splice sites, or can interfere with the binding of proteins bound to enhancers. Recently, hnRNP proteins have been shown to hinder communication between factors bound to different splice sites. Conversely, several reports have described a positive role for some hnRNP proteins in pre-mRNA splicing. Moreover, cooperative interactions between bound hnRNP proteins may encourage splicing between specific pairs of splice sites while simultaneously hampering other combinations. Thus, hnRNP proteins utilize a variety of strategies to control splice site selection in a manner that is important for both alternative and constitutive pre-mRNA splicing.

Patient 4 had a heterozygotic 718G > C transition in exon 4, which results in a replacement of alanine by proline at the codon 240. This mutation has been reported to be polymorphic (rs4904210) in many African, American, and European subpopulations (Table 5). We do not know anything about potential significance of this mutation for the structure and function of the PAX9 protein and further studies are needed to establish the presence or absence of an association between this mutation and the PAX9 phenotype. We can only speculate that because proline is an amino acid, which has a unique structure because of the bonds between its side chain and nitrogen atom of the main chain and α-carbon, its presence in the

Table 5. **Mutations in the PAX9 gene in various populations**

Mutation		Population	Population diversity					Ref.
IVS2-109G > C			GG	GC	CC	G	C	
		Polish patients H/O	9 (0.24)	15 (0.39)	14 (0.37)	33 (0.43)	43 (0.57)	(Present study)
		Healthy Polish	13 (0.13)	59 (0.59)	28 (0.28)	85 (0.42)	115 (0.58)	(Present study)
IVS2-54A > G			AA	AG	GG	A	G	(Present study)
		Polish patients H/O	9 (0.24)	15 (0.39)	14 (0.37)	33 (0.43)	43 (0.57)	(Present study)
		Healthy Polish	6 (0.06)	60 (0.60)	34 (0.34)	72 (0.36)	128 (0.64)	(Present study)
IVS2-41A > G			AA	AG	GG	A	G	
		Polish patients H/O	15 (0.39)	17 (0.45)	6 (0.16)	47 (0.62)	29 (0.38)	(Present study)
		Healthy Polish	29 (0.29)	57 (0.57)	14 (0.14)	115 (0.58)	85 (0.42)	(Present study)
IVS3+40G > A			GG	GA	AA	G	A	
		Polish patients H/O	3 (0.43)	2 (0.29)	2 (0.29)	8 (0.57)	6 (0.43)	(Present study)
717C > T	rs12881240		CC	CT	TT	C	T	
	His239His	African-American	52 (0.90)	**0 (0.00)**	**6 (0.10)**	**104 (0.9)**	**12 (0.1)**	(NCBI)
		Polish patients H/O	2 (0.33)	2 (0.33)	2 (0.33)	6 (0.50)	6 (0.50)	(Present study)
718G > C	rs4904210		GG	CG	CC	G	C	
	Ala240Pro	African-American	50 (0.86)	0 **(0.00)**	8 (0.14)	100 (0.86)	16 (0.14)	(NCBI)
		Native Americans	52 (0.91)	5 (0.09)	0 (0.00)	109 (0.96)	5 (0.04)	(32)
		Asians	11 (0.79)	2 (0.14)	1 (0.07)	24 (0.86)	4 (0.14)	(32)
		Europeans	10 (0.67)	3 (0.20)	2 (0.13)	23 (0.77)	7 (0.23)	(32)
		Polish patients H/O	22 (0.42)	29 (0.56)	1 (0.02)	73 (0.70)	31 (0.30)	(32)
		Polish patients CL/P	77 (0.50)	67 (0.44)	10 (0.06)	221 (0.72)	87 (0.28)	(32)
		Healthy Polish	60 (0.42)	73 (0.51)	11 (0.07)	193 (0.67)	95 (0.33)	(32)
		Polish patients H/O	5 (0.83)	1 (0.17)	0 (0.00)	11 (0.92)	1 (0.08)	(Present study)
		Healthy Chinese	35 (30.2)	52 (44.8)	29 (25.0)	122 (0.53)	110 (0.47)	(33)
		Chinese patients H/O	29 (28.4)	45 (44.1)	28 (27.5)	103 (0.50)	101 (0.50)	(33)
1579G > C			GG	GC	CC	G	C	
		Polish patients H/O	4 (0.67)	2 (0.33)	0 (0.00)	10 (0.83)	2 (0.17)	(Present study)
1843 C > T			CC	CT	TT	C	T	
		Polish patients H/O	3 (0.50)	2 (0.33)	1 (0.17)	8 (0.67)	4 (0.33)	(Present study)
A > G	rs2073244		AA	AG	GG	A	G	
		Healthy Chinese	33 (28.4)	55 (47.4)	28 (24.2)	121 (0.52)	111 (0.48)	(33)
		Chinese patients H/O	27 (26.4)	46 (45.1)	29 (28.5)	100 (0.49)	104 (0.51)	(33)
C > G	rs2073245		CC	CG	GG	C	G	
		Healthy Chinese	36 (31.0)	52 (44.8)	28 (24.2)	124 (0.53)	108 (0.47)	(33)
		Chinese patients H/O	28 (27.5)	47 (46.1)	27 (26.4)	103 (0.50)	101 (0.50)	(33)
C > T	rs2073247		CC	CT	TT	C	T	
		Healthy Chinese	26 (22.4)	57 (49.1)	33 (28.5)	109 (0.47)	123 (0.53)	(33)
		Chinese patients H/O	27 (26.4)	40 (39.2)	35 (34.4)	94 (0.46)	110 (0.54)	(33)

H/O, hypodontia/oligodontia; CL/P, cleft lip/palate.

mutated PAX9 protein may likely affect its secondary structure. Patients 5 and 6 had a homozygotic 717C > T transition, being a silent His239 mutation. This mutation was reported as a polymorphism in a small African-American population (rs12881240) with the distribution of genotypes C/C 0.9 and TT 0.1 (Table 5). In this case, a codon was changed into a synonymous codon. The degeneration of the genetic code and the

lack of reverse translation of proteins cause ambiguity in the codon usage. Synonymous codons are not equally used in different genomes, and even in the single genome the synonymous codon usage can vary widely among genes or along the gene sequences. Unequal usage of synonymous codons is called codon bias and is frequently explained by the mutation–selection balance (28).

Codon usage and nucleotide content at fourfold degenerated positions are not uniform across eukaryotic exons. In humans, the variation of nucleotide contents is consistent with the distribution of known enhancer elements (29). Selection at the RNA processing level – splice sites recognition and selection – contributes to the codon bias in the human genome. In most cases, hypothetical reverse translation uses the codon usage statistics of the complete genome or a representative set of genes for the organism of interest and may depend on sequences flanking this codon (30). Silent mutations are subjected to constrain, often because they affect splicing and/or mRNA stability (31). Apart from the codon bias, silent mutations in exons can affect the function of splicing regulatory elements such as sequences of splicing enhancers and silencers.

To check whether the IVS2-54A > G, IVS2-109G > C, and IVS2-41A > G mutations are associated with oligodontia, we compared their frequency in the cohort of 38 patients with oligodontia and 100 controls without tooth agenesis. We observed a higher frequency of the wild-type alleles of the IVS2-54A > G and IVS2-109G > C mutations in patients with oligodontia than in the controls. Therefore, we detected some mutations, which are listed in data bases as polymorphisms and have their rs numbers. However, remaining mutations are not mentioned in any known base. A further study with a larger sample size is needed to check whether these mutations may be polymorphisms in Polish population.

Conclusion

The IVS2-54A > G, IVS2-109G > C, and IVS2-41A > G mutations in the PAX9 gene can be polymorphisms in the Polish population and can be associated with sporadic oligodontia, but further studies on the structure and functional significance of the gene and on the pathogenesis of the anomaly are needed to establish the association between the PAX9 genotype and oligodontia prevalence.

Clinical relevance

Because tooth development includes a series of genetic interactions, abnormal development may be the result of the mutations in genes, which play a role in dental development. Knowledge of such mutations is important for clinical practice, as it enables better diagnosis of oligodontia. We identified several mutations in the PAX9 gene, encoding a protein, which is important in tooth development, and some of them may be polymorphic and so requiring relatively simple techniques to be detected.

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