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ORIGINAL ARTICLE

Novel PAX9 gene polymorphisms and mutations and susceptibility to tooth agenesis in the Czech population

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Abstract

OBJECTIVES: Tooth agenesis is one of the most common developmental anomalies in humans. Genetic and environmental factors may be of etiological importance in this condition. Among genes involved in tooth morphogenesis, mutations in PAX9, MSX1, AXIN2, WNT10a, and EDA genes have been associated with tooth agenesis. The aim of our study was to investigate the relationship between the PAX9 gene variants and tooth agenesis in the Czech population.
METHODS: The selected regions of the PAX9 gene were analysed by direct sequencing and compared with the reference sequence from the GenBank online database (NCBI).
RESULTS: We found several novel variants in the PAX9 gene, e.g. insertion g.5100_5101insC (rs11373281) with simultaneous substitution g.5272C>G (rs4904155) in exon 1, and mutation g.10934C>T (Gly203Gly, rs61754301) in exon 3. In subjects with full dentition we observed polymorphisms g.10276A>G (rs12882923) and g.10289A>G (rs12883049) in IVS2 (intervening sequence 2) previously related to tooth agenesis in Polish study.
CONCLUSIONS: In our study we excluded a direct effect of rs12882923 and rs12883049 polymorphisms on the dental agenesis in the Czech population. All described PAX9 genetic variants were present both in patients with tooth agenesis and controls. We expect that tooth agenesis in our cohort of patients is caused by mutations in regions different from PAX9 exons analyzed in our study.

Abbreviations:

AXIN2	- axis inhibitor 2
BMP	- bone morphogenetic protein
DMSO	- dimethyl sulfoxide
DNA	- deoxyribonucleic acid
EDTA	- ethylenediaminetetraacetic acid
FGF	- fibroblast growth factor
Msx1	- muscle segment homeobox 1
ncRNA	- non-coding RNA
PAX9	- paired box gene 9
PCR	- polymerase chain reaction
SHH	- sonic hedgehog
tRNA	- transfer RNA
UTR	- untranslated
Wnts	- wingless-type MMTV integration site family

INTRODUCTION

Odontogenesis is a very complicated and complex process involving over 350 so far identified proteins that interact with each other (Lin *et al.* 2007; Matalová *et al.* 2008). Even a very small change in the protein or regulatory process involved in the spatio-temporal cohesion between the individual protein interactions during tooth development can result in tooth agenesis (Mues *et al.* 2009).

Tooth agenesis is the most prevalent craniofacial congenital malformation in humans (Šerý *et al.* 2013). Up to 25 % of the population may lose at least one third molar. Agenesis of other permanent teeth, excluding third molars, ranges from 1.6 to 9.6 %, depending on the population studied. Second premolars and upper lateral incisors are predominantly affected. Primary dentition may also be affected, but with lower prevalence (from 0.5 to 0.9 %), (ref. Matalová *et al.* 2008).

Tooth development begins in the early stages of embryonic development and comes through four main stages. First, the epithelium thickens to form a dental placode as an early signalling centre. Then, the epithelium grows into the underlying mesenchyme and forms a tooth bud. Subsequently, the mesenchyme condenses, the oral epithelium overgrows it and forms a cup stage that is characterized by the presence of the primary enamel knot. Epithelial cells still proliferate, secondary enamel knots are formed and a bell is formed (Chinsembu 2012; Klein *et al.* 2006; Peterkova *et al.* 2014; Thesleff 2003).

During key morphogenetic steps of tooth development, the appearance of transient epithelial structures, signalling centres, is a characteristic feature of odontogenesis. These signalling centres express signal molecules including BMPs (bone morphogenetic protein), SHH (sonic hedgehog), FGFs (fibroblast growth factor), and Wnts (wingless-type MMTV integration site family) that are responsible for the maintenance of signalling centre function, proliferation of surrounding epithelial and mesenchymal cells, morphogenesis of the tooth germ and terminal self-elimination by apoptosis (Fleischmanová *et al.* 2007; Peters *et al.* 1998; Thesleff *et al.* 2001).

PAX9 gene (Paired box 9) is a member of the PAX family (Paired box domain gene family) that is characterized by a common motif – the DNA-binding domain. It is encoded by the paired box, a conserved DNA region originally identified in *Drosophila*. In mammals, nine different PAX genes that belong to four different subgroups have been isolated (Peters *et al.* 1998). Human PAX9 gene that encodes *Pax9* protein is located on chromosome 14 (14q-12-q13) and contains 5 exons. *Pax9* protein acts as an important transcription factor for embryogenesis and participates in the differentiation and maintenance of pluripotence of cell populations (Gerits *et al.* 2006; Klein *et al.* 2005; Krejčí *et al.* 2007).

PAX9 gene expression is a marker of an early odontogenesis phase and it occurs before the expression of other genes (Mensah *et al.* 2004). *Pax9* is essential for progressive and reciprocal interactions between oral epithelium and neural crest-derived mesenchyme (Gerits *et al.* 2006; Krejčí *et al.* 2007). *Pax9* is essential for dental mesenchyme condensation (Krejčí 2006). The mesenchymal cells condense around the overgrown epithelium. In the early stages of tooth development, *Pax9* is expressed in the dental mesenchyme under control of Fgf8/Bmp4 antagonistic signalling (Krejčí *et al.* 2007). Once the basic dentition pattern has been established, the expression of Fgf8 in the epithelium induces the expression of *Pax9* necessary for tooth development. On the other hand, *Bmp4* inhibits *Pax9* expression in presumptive toothless sides (Fleischmanová *et al.* 2007). In the bud stage and during the transition to the cap stage, *Pax9* interacts with *Msx1* (Muscle segment homeobox 1) and *Bmp4* (Bone morphogenetic protein 4) (Matalová *et al.* 2008; Peters *et al.* 1998). This is the key event for tooth germ development because *Bmp4* is is necessary for production of primary enamel knot (Chinsembu 2012). Impaired *Pax9* function then leads to a decreased expression of crucial odontogenic molecules in the mesenchyme. Tooth development cannot continue to the next stage and odontogenesis is inhibited at the bud stage (Krejčí 2006). High *Pax9* expression persists in the dental mesenchyme during the bud and cup stages then during the bell stage (Mensah *et al.* 2004) it decreases.

Mutation and/or polymorphisms in PAX9 gene may affect DNA binding ability, transcriptional activity and synergistic interactions with co-activators, such as *Msx1* (Wang *et al.* 2009) and lead to tooth agenesis. To date over 30 polymorphisms in the PAX9 gene have been associated with tooth agenesis. However, published results are inconsistent and differ between the individual studies/populations. The aim of our study was to investigate the relationship between the PAX9 gene variants and tooth agenesis in the Czech population.

MATERIALS AND METHODS

The sample consisted of 97 patients (59 women and 38 men) with tooth agenesis and 20 subjects with complete dentition as the control group. The age of patients and controls ranged from 9 to 65 years. Diagnosis of tooth agenesis was performed using X-ray images. Samples were obtained from Dental Clinics in Brno (Assoc. Prof. Černochová P., MD., PhD.), Ostrava (Štembírek J., MD., PhD.) and Olomouc (Krejčí P., MD., PhD.). Study was performed with the approval of the Ethics Committee of St. Anne's University Hospital Brno. Written informed consent was obtained from all participants before inclusion in the study, in line with the Helsinki declaration. DNA was isolated from buccal swabs with automated magnetic bead-based protocol of the chemagic Prepito (Perkin Elmer, USA). The quality and concentration of DNA were checked by spectrophotometric measurement. Genomic DNA was used for capillary DNA sequencing using the automated ABI 3130 Genetic Analyzer (Applied Biosystems, USA).

We selected the first three exons and adjacent intronic regions to determine the presence of mutations and/or polymorphisms in the PAX9 gene. Exon 1 of the PAX9 gene is not translated (5'-UTR region), but it may influence translation. Exon 2 is a next part of the 5'-UTR region and at the end of this region there are four bases that are translated (i.e. the initiation codon and the first base of the next encoding triplet). Entire exon 3 is translated and encodes a DNA-binding domain. The intronic regions in the adjacent coding regions were also analyzed as they may have a significant influence on the regulation of gene expression through ncRNA (non-coding RNA) (Šerý *et al.* 2013).

Exons 1–3 of PAX9 gene were amplified by PCR in a 20 µl reaction volume. Specific PCR primers were used for DNA amplification (Table 1). The PCRs of exons 1 and 3 of the PAX9 gene were performed with the KAPA2G Fast HotStart ReadyMix (Kappa Biosystems, USA). For exon 2 of the PAX9 gene, KAPA2G Robust HotStart kit (Kapa Biosystems, USA) + 5% DMSO was used. The conditions for PCR were: 95°C for 2.5 min activation/denaturation step, followed by 45 cycles of 95 °C for 30 s, 58 °C (for exon 1 and 3) or 54 °C (for exon 2) for 30 s, 72 °C for 60 s, with a final extension for 7 min. The Veriti thermal cycler (Applied Biosystems, USA) for all PCR reactions was used.

Amplicons were purified by ExoI-FastAP (Fermentas, USA). The mixtures were incubated at 37 °C for 15 min and at 85 °C for 15 min to inactivate the enzymes followed by sequencing with BigDye Terminator v.3.1 (Applied Biosystems, USA). Sequencing reactions were purified by EDTA/ethanol precipitation, resuspended in 10 µl Hi-Di Formamide (Applied Biosystems, USA), and sequenced on an automated ABI 3130 Genetic Analyzer (Applied Biosystems, USA).

Finally, sequences were compared by BioEdit v.7.0.8.0 (Hall 1999) with standard sequence NG_013357.1 of PAX9 gene obtained from the GenBank database and their possible effects on the gene expression or protein sequence were described.

RESULTS

In our sample of 97 patients, the most frequently missing teeth were the third molars, followed by the second lower premolars and the second upper incisors. We observed several novel mutations or polymorphisms in the PAX 9 gene (Figure 1, Table 2).

Heterozygous/homozygous insertion of cytosine at the nucleotide position g.5100_5101insC (rs11373281) in exon 1 was found in patients with tooth agenesis. The same insertion was present in control subjects with complete dentition.

The heterozygous substitution g.5272C>G (rs4904155) occurred simultaneously with the insertion g.5100_5101insC in four samples and the combination of the homozygous insertion g.5100_5101insC occurred simultaneously with the homozygous substitution at position g.5272C>G (rs4904155) in 19 samples. Agenesis of the third molars in the first quadrant, and agenesis of the third molars in the remaining quadrants were the most common anomaly in this group of 19 subjects. Mandibular second molars and mandibular second premolars in the third and fourth quadrant were further frequently missing teeth.

The insertion g.5100_5101insC and the polymorphism g.10288A>C occurred in 3 patients. The heterozygous insertion g.5100_5101insC occurred with the heterozygous substitution g.5272C>G and g.10288A>C, but only in a few sporadic cases.

We found no polymorphism in exon 2 in patients or controls, all subject carried just the standard sequences.

Tab. 1. Sequences of primers used for PCR amplification and sequencing reaction of human PAX9 exons.

Gene	Exon	PCR amplification		Sequencing reaction
		Forward primer	Reverse primer	
PAX9	1	5´-cagaaagtaatgttagggtcacg-3´	5´-caagtgacagccagaagctc-3´	5´-tgcttatatgctcggaaaac-3´
	2	5´-ctcccacctatagccttaacttc-3´	5´-agctccctctcttaaaatcaga-3´	5´-tcgagtcattcacattcaga-3´
	3	5´-gattggacagtgacggtttg-3´	5´-ggaaagacagtgtccctgag-3´	5´-ggacagccccagtagttagt-3´

Tab. 2. The description of PAX9 mutations and polymorphisms described in the study and their comparison with the DNA databases.

dbSNP rs# cluster id[1]	Position in the whole gene region[2]	Exon position		CDS position	Amino acid change[3]
rs11373281	g.5100_5101insC	100_101insC	Exon 1	-/5´-UTR	-
rs4904155	g.5272C>G	272C>G		-/5´-UTR	-
rs12882923	g.10276A>G	-54A>G	IVS2	-/intron	-
rs12883049	g.10289A>G	-41A>G		-/intron	-
none	g.10288A>C	-40A>C		-/intron	-
rs61754301	g.10934C>T	c.605C>T	Exon3	c.609C>T	Gly203Gly

[1]According to NCBI dbSNP Short Genetic Variations: id 5083
(http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?showRare=on&chooseRs=all&go=Go&locusId=5083)
[2]According to NCBI Reference Sequence: NG_013357.1 (http://www.ncbi.nlm.nih.gov/nuccore/262331554)
[3]According to NCBI CCDS Database: CCDS9662.1
(http://www.ncbi.nlm.nih.gov/CCDS/CcdsBrowse.cgi?REQUEST=CCDS&DATA=CCDS9662.1)

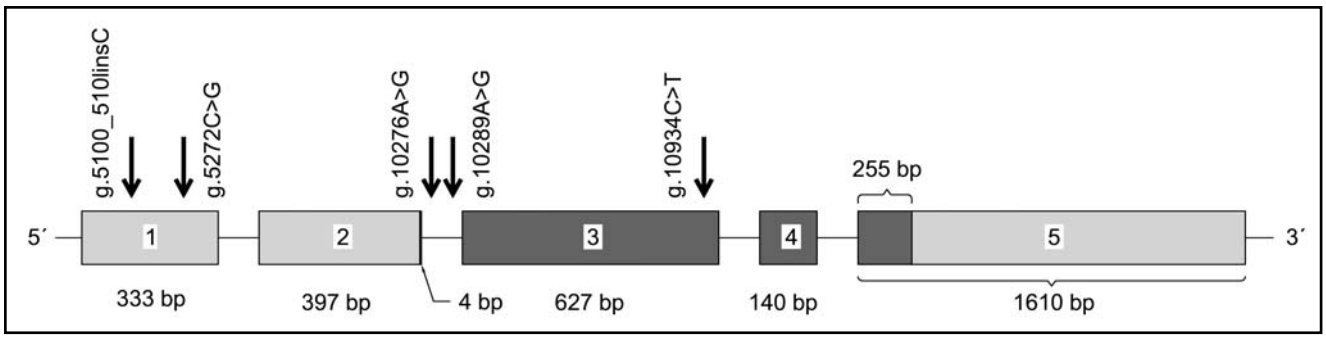

Fig. 1. Schematic illustration of the PAX9 gene and found mutations grey colour – untranslated region (UTR); dark grey colour – coding sequence (CDS).

The polymorphisms g.10276A>G (rs12882923) and g.10289A>G (rs12883049), both individually and in combinations, both in patients and in control subjects, were the most frequent polymorphisms in exon 3. Both polymorphisms are located in the noncoding region before exon 3 at position distant 54, and 41 bp from the first nucleotide of exon 3.

In three families, we found the mutation g.10934C>T (rs61754301) located at the coding nucleotide position 605. The coded 203rd amino acid remains glycine. Other sequences were aligned as standard.

DISCUSSION

Pax9 is a transcriptional factor that regulates expression of key odontogenic molecules in the mesenchyme during the dental bud stage and enables the transition of the tooth germ to the next cup stage. Disruption of PAX9 gene function causes inhibition of odontogenesis at the bud stage. In this study we sequenced selected regions of the PAX9 gene and examined possible relationships between PAX9 gene variants and tooth agenesis.

The combination of homozygous cytosine insertion g.5100_5101insC with the homozygous substitution g.5272C>G were the most frequently found polymorphisms in the group of patients (19 of 97 cases, i. e. 19.6%). Both polymorphisms are located in the 5'-untranslated region (5'-UTR), which may influence transcription, post-transcriptional RNA modifications, translation (Mignone *et al.* 2002; Wang *et al.* 2005) or microRNA functions (Cao *et al.* 2010; Lee *et al.* 2009). Most patients suffered from agenesis of the third molars, mandibular second molars or mandibular second premolars. We found the heterozygous variants of these two simultaneously located polymorphisms both in patients and in persons with complete dentition, therefore we could not decide if these polymorphisms represent just interindividual variance without significant consequences. Interestingly, these two polymorphisms always occurred in the sequence of PAX9 gene simultaneously in all cases. In the patient group, besides the above-mentioned polymorphisms, in a few sporadic cases, we also found other substitutions and combinations, however, their possible relationships with tooth agenesis cannot be accepted without confirmation in a larger group of patients.

To the best of our knowledge, no study analysing exon 1 of the PAX9 gene has been published. Further,

we found incorrect numbering of exons in several scientific papers, identifying exon 3 as exon 2.

The most interesting finding was the mutation g.10934C>T (Gly203Gly) in the coding region (in the third exon) of the PAX9 gene totally in six patients and controls from three families. In the first family, this mutation was present in a male patient with missing both second premolars in the lower jaw. His mother has the same mutation missing just third molars in the upper jaw. In the second family, we found this mutation in both healthy parents – father with full dentition (Figure 2), mother with missing all third molars (Figure 3), and in healthy sister (with missing all third molars, Figure 4) of female proband with tooth agenesis (missing both first incisors in the lower jaw, Figure 5). In this female proband we did not find any mutation. In the third family, mutation was found only in father (full dentition) of male proband with oligodontia (totally 17 missing teeth including third molars). Mutation g.10934C>T (Gly203Gly) does not change sense codon but different tRNA is necessary to recognize this codon. In the standard PAX9 gene sequence, we found totally 30 codons for glycine: 6 codons GGT, 11 codons GGC, 6 codons GGA, and 7 codons GGG. In case of g.10934C>T (605 Y, Gly203Gly, rs61754301) mutation codon GGC is changed to codon GGT. We do not presume this mutation to be associated with tooth development due to its presence in both healthy and patients with tooth agenesis

The intron substitutions g.10289A>G and g.10276A>G were identified in the PAX9 gene. Polish study published by Pawlowska *et al.* (2010) associated these two polymorphisms with tooth agenesis. In our study we found both polymorphisms only in persons with complete dentition, therefore it is possible to exclude a direct effect of these polymorphisms on the dental agenesis in the Czech population.

Our findings do not fully confirm results of other studies carried out on the PAX9 gene. We paid special attention to exon 3 because DNA-binding domain coded in this area allows the protein to act as a transcription factor; disruption of its function is generally regarded as the main pathological cause of tooth agenesis. Although several polymorphisms have been described in exon 3, these data have not been confirmed in the Czech population and on the other hand, we detected polymorphisms not described in any of the studies. This discrepancy could be caused by a genetic difference of Czech people with tooth agenesis versus previously studied populations (Gerits *et al.* 2006; Klein *et al.* 2005; Mensah *et al.* 2004; Pawlowska *et al.* 2010; Zhao *et al.* 2007; Wang *et al.* 2009). Standard PAX9 nucleotide sequences also occurred in patients with confirmed dental agenesis, implying thus multifactorial etiopathogenesis of this condition. Tooth development is controlled by expression of a large number of known genes (e.g. MSX1 and AXIN2). These genes were not analyzed in this study and can be a cause of tooth agenesis in our patients.

In contrast to genetic data, the most frequently missing teeth were molars (mostly third molars) followed by premolars. These results are in agreement with the find-

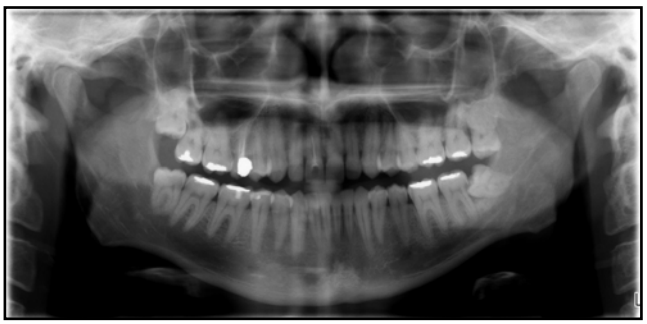

Fig. 2. Panoramic radiograph of unaffected father proband (full dentition, dislocation 38).

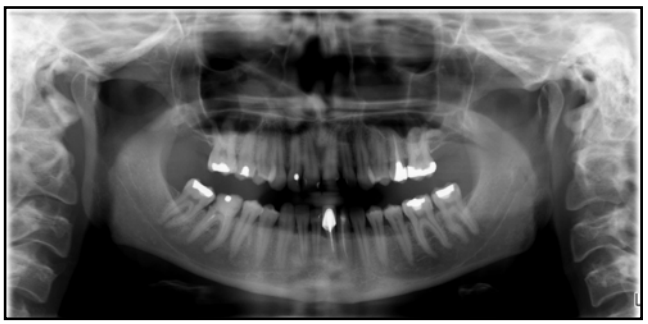

Fig. 3. Panoramic radiograph of unaffected mother proband (full dentition, condition after tooth extraction 17, 27).

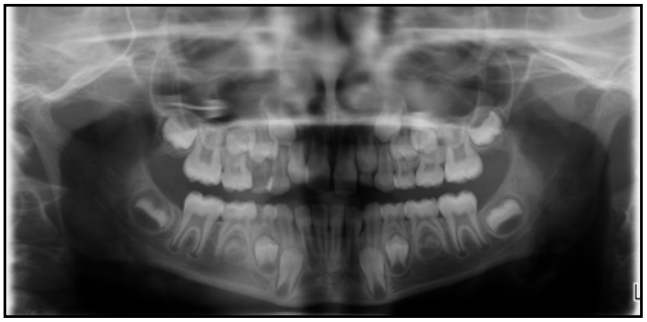

Fig. 4. Panoramic radiograph of unaffected sister proband (susp. agenesis 18, 28, 38, 48).

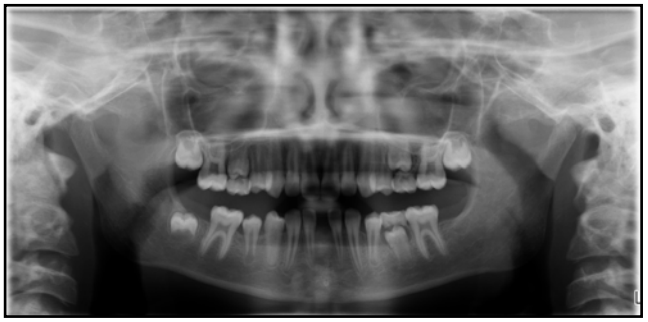

Fig. 5. Panoramic radiograph of female proband (agenesis 18, 28, 38, 37, 31, 41, 48).

ings of other studies from different populations (Symons *et al.* 1993; Závadová 2002; Heringová & Černochová 2009). In our group of patients, the bilateral absence of teeth occurred very often, this is in compliance with the previous Czech and foreign studies (e.g. Symons *et al.* 1993; Závadová 2002; Heringová & Černochová 2009). In addition, discrepancies among the individual studies can be caused by a different sex representation. A higher incidence of tooth agenesis for females than males (1.37 times higher) has been reported (Zhao *et al.* 2007). Although our group of subjects was not chosen randomly, the ratio of females to males approximately equalled to the value published in papers (i.e. 3:2).

CONCLUSION

In conclusion, we found insertion g.5100_5101insC with simultaneous substitution g.5272C>G (rs4904155, heterozygous and homozygous) in exon 1 and polymorphisms g.10276A>G (rs12882923), g.10289A>G (rs12883049) and mutation g.10934C>T (Gly203Gly, rs61754301) in exon 3 of the PAX9 gene in the Czech sample of patients with tooth agenesis and healthy controls. These polymorphisms cannot be considered a cause of tooth agenesis and should be further studied in relationship with results of the sequence analysis of other candidate genes, such as as MSX1 and AXIN2.

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