Příloha I – *PAX9* gene mutations and tooth agenesis: A review

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REVIEW

PAX9 gene mutations and tooth agenesis: A review

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Paired box 9 (PAX9) is one of the best-known transcription factors involved in the development of human dentition. Mutations in *PAX9* gene could, therefore, seriously influence the number, position and morphology of the teeth in an affected individual. To date, over 50 mutations in the gene have been reported as associated with various types of dental agenesis (congenitally missing teeth) and other inherited dental defects or variations. The most common consequence of *PAX9* gene mutation is the autosomal-dominant isolated (non-syndromic) oligodontia or hypodontia.

In the present review, we are summarizing all known *PAX9* mutations as well as their nature and precise loci in the DNA sequence of the gene. Where necessary, we have revised the loci of the mutations in line with the reference sequence of the *PAX9* gene as it appears in the current DNA databases.

KEYWORDS

PAX9, gene, hypodontia, oligodontia, mutation

1 | INTRODUCTION

The paired box (PAX) family of transcription genes plays an important role in the mechanisms of ontogeny in all classes of vertebrates.[1] Recent review identifies *PAX9* as one of the most widely studied gene participating in odontogenesis.[2] Therefore, the present review focuses on associations between mutations in *PAX9* and occurrence of CMT (congenitally missing teeth) and/or other variations in human teeth. When appropriate, we offer a critical appraisal of the studies and suggest future directions.

In humans, *PAX9* gene is located on the long arm of chromosome 14 (14q13.3). The structure of the gene consists of 5 exons. The first exon represents only 5′-untranslated region (UTR) while the following exons 2 to 5 contain 1026 bases (including the stop codon) encoding 341 amino acids constituting the human PAX9 protein. The initiation codon and the first base of the second triplet are the only coding elements of the second exon. The rest of the second exon is also a UTR. The most interesting protein element—the paired domain—is encoded in the third exon. The rest of the coding sequence and 3′-UTR are located in fourth and fifth exon (Figure 1).

The large screen of *PAX9* gene sequences from various vertebrates species such as *Chiroptera* (bats), *Mus musculus*, *Canis familiaris*, *Felis catus*, *Homo sapiens* revealed 2 conserved islands—a region of 146 bp situated 113 bp from the start codon in the 5′-UTR sequence of the first exon and a region of 204 bp placed within the 3′-UTR sequence 196 bp downstream of the stop codon. The third exon (coding paired domain) is the most conserved region of the *PAX9* gene.[3]

2 | MUTATIONS AND POLYMORPHISMS IN THE *PAX9* GENE

To date, more than 50 mutations in intron (IVS—intervening sequence) and exon regions (both coding and UTRs) have been found. Most of these are missense mutations or insertions/deletions causing amino acid substitution in the most important region—paired domain of the PAX9 protein (1st to 136th amino acid).

PAX9 gene also contains silent mutations (synonymous mutations) associated with CMT. These silent mutations in codons are not causing changes in the structure of the translated proteins because they do not influence the sense of amino acid coding. The phenomenon of multiple (synonymous) codons encoding the same amino acid is called codon usage bias. Changes to codon usage bias would alter

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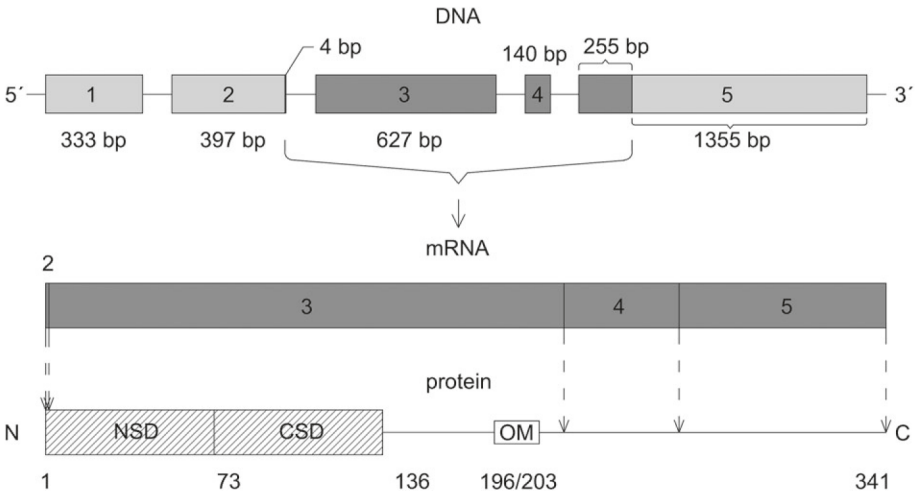

FIGURE 1 Schematic description of nucleotide sequence, mRNA and protein structure of the PAX9 gene. Light grey colour shows untranslated region (UTR); dark grey colour shows coding sequence (CDS); NSD, N-terminal subdomain; CSD, C-terminal subdomain; OM, octapeptide motif

the pattern of tRNA's used in the translation and, in theory, this could accelerate/decelerate the protein synthesis. However, it is well known that, at least in *Homo sapiens*, the availability of tRNA has only limited effectiveness on the rate of translation and does not significantly influence the amount of the produced protein.[4]

In the next section, we shall proceed from the first to the fifth exon and discuss the most interesting cases of mutations and polymorphisms (focusing mainly on the reports that are accompanied by physiological studies). In the section, which follows we shall look at the changes in intronic sequences and, finally, we shall address the subject of the deletion whole *PAX9* gene.

All mutations showed in Table 1 and in the text have been revised and, when needed, their locations corrected to put them in line with the reference sequence NG_013357.1 of the *PAX9* gene as it currently appears in the NCBI database (detailed in Table 1 legend). In Table 1, publications with alternative exon numbering or incorrect (with respect to the above criteria) locations of nucleotides are marked with asterisks.

2.1 | Exon 1

There has been little attention given to the exon 1 probably because it contains only UTR sequences, which are neither transcribed nor translated. Four studies, including 2 from our laboratory (see Table 1), detected 2 mutations in this region. In particular, we have found rather intriguing simultaneous appearances of the g.5100_5101insC and g.5272C>G polymorphisms, both always present in the same allelic form (homozygous/heterozygous state) but neither having any known effects on tooth agenesis.[6,7] The polymorphism G.5272C>G (rs4904155) was, however, significantly associated with the crown size and shovelling of the upper first incisor. In Asian population, significant differences between the frequencies of rs4904155 polymorphism in controls and patients with hypodontia have been reported.[5,8]

2.2 | Exon 2

The second exon of the *PAX9* gene is constituted almost entirely of UTR sequences except at the end where the first (initiation) codon and the first base of the second triplet are situated. Two causative mutations affecting initiation of translation have been detected in this exon. Klein et al[15] reported g.9523A>G mutation and Liang et al[16] found g.9524T>G mutation. Both authors suggested haploinsufficiency of *PAX9* gene because of impossibility of the ribosome to initiate translation of the affected mRNA. In both cases, this resulted in non-syndromic oligodontia with similar patterns of missing teeth in the affected families. Most of the individuals lacked the first or second premolars and all permanent molars. In some individuals, at least one of the canines was missing and some teeth were "peg shaped". One of the families had only their permanent teeth affected with no apparent defects in the primary dentition.[16]

2.3 | Exon 3

It is no coincidence that the first mutation in *PAX9* gene associated with oligodontia[34] was found in exon 3; this sequence represents by far the largest coding region of the gene. Stockton et al[34] enrolled 47 members of an affected family and found that probands with oligodontia carried an insertion of guanine at position g.10544. This is a frameshift mutation resulting in a premature termination of the PAX9 protein synthesis at the position 316 in the amino acid sequence. Stockton et al[34] suggested that it may have influenced an amino acid sequence within the DNA-binding domain and completely and irreversibly abolish the regulatory function of the protein. Four years later, Mensah et al[35] made in vitro functional analysis of non-mutant and 219insG versions of PAX9 gene and described a decreasing binding activity and transactivation potential of mutated protein and also changes in its nuclear localization. These findings appear to be in accordance with the proposed loss of function of PAX9 protein resulting in haploinsufficiency thus plausibly explaining the altered odontogenesis.

Another in vitro study using the PAX9 protein comprising a mutated paired domain with amino acid change Arg28Pro revealed dramatically reduced ability to form protein-DNA complex.[28] Wang et al[20] made functional analysis on Gly6Arg and Ser43Lys substitutions and reported no effect on nuclear localization of PAX9 but found reduced DNA-binding activity of the mutant protein compared to the wild type. These findings were provided with additional support by observations of Zhao et al[29] who studied Arg47Trp amino

TABLE 1 Summary of mutations in the PAX9 gene

PAX9						
dbSNP rs# cluster id[a]	Position in whole-gene region[b]	CDS/exon position[c]		Amino acid change[c]	Phenotype	Reference, notice
rs2295222	g.4536C>A	−1191C>A	5´-near gene	Intron	NSA with crown size and third molar agenesis	5
rs11373281	g.5100_5101insC	−628_-627insC	EXON 1	5′-UTR	Hypo-/oligodontia, NSA	6,7
rs4904155	g.5272C>G	−455C>G		5′-UTR	Molar agenesis, NSA Sporadic non-syndromic hypodontia Hypo-/oligodontia	5–8
rs2073244	g.8102A>G	−1028A>G	INTRON 1 (IVS1)	Intron	Various type of hypodontia Sporadic tooth agenesis, NSA Molar agenesis, NSA Sporadic non-syndromic hypodontia, NSA Non-syndromic hypodontia Non-syndromic hypo-/oligodontia	9[d] 5,8,10,11[d] 12
rs2073245	g.8158C>G	−972C>G		Intron	Third molar agenesis	13[d]
rs2073246	g.8221C>T	−909C>T		Intron	Various type of hypodontia	9[d] 11[d] 12
None	g.8264G>A	−866G>A		Intron	Autosomal-dominant molar hypodontia	14
rs2073247	g.8973C>T	−157C>T		Intron	Sporadic non-syndromic hypodontia Sporadic tooth agenesis	8,10
None	g.9523A>G	1A>G		Met1Val	Autosomal-dominant molar oligodontia (+second premolars)	15[d]
None	g.9524T>G	2T>G	EXON 2	Met1Arg	Autosomal-dominant non-syndromic oligodontia	16[d]
None	g.9527G>T	−695G>T	INTRON 2 (IVS 2)	Intron, splicing site	Autosomal-dominant, non-syndromic oligodontia	7
rs12883298	g.10221G>C	−109G>C		Intron	Sporadic non-syndromic oligodontia Autosomal-dominant non-syndromic hypo-/oligodontia Non-syndromic hypo-/oligodontia	17,18[d] 19
rs12882923	g.10276A>G	−54A>G		intron	Sporadic non-syndromic oligodontia Hypo-/oligodontia, NSA Autosomal-dominant non-syndromic hypo-/oligodontia Non-syndromic hypo-/oligodontia	6,17,18[d] 19
rs12883049	g.10289A>G	−41A>G		Intron	Sporadic non-syndromic oligodontia Hypo-/oligodontia, NSA Non-syndromic hypo-/oligodontia	6,17,19
None	g.10341G>A	16G>A		Gly6Arg	Mand. central incisors, max. second premolar	20[d]
None	g.10368T>A	43T>A		Phe15Ile	Autosomal-dominant non-syndromic oligodontia	21
None	g.10672A>T	47A>T		Asn16Ile hom.	Sporadic non-syndromic hypodontia	22
None	g.10384delC	59delC		Pro20ArgfsX65	Autosomal-dominant syndromic oligodontia, trychodysplasia	23[d]
None	g.10384C>T	59C>T		Pro20Leu	Autosomal dominant oligodontia	24[d]
rs28933970	g.10387T>C	62T>C		Leu21Pro	Autosomal-dominant hypodontia	25[d]
None	g.10398_10400delATC	73_75delATC		Ille25del	Sporadic non-syndromic oligodontia	26[d]

(*Continues*)

TABLE 1 (Continued)

PAX9					
dbSNP rs# cluster id[a]	Position in whole-gene region[b]	CDS/exon position[c]	Amino acid change[c]	Phenotype	Reference, notice
None	g.10400C>T	75C>T	Ile25Ile	Autosomal dominant oligodontia	24[d]
rs28933972	g.10401C>T	76C>T	Arg26Trp	Autosomal-dominant oligodontia with reduced size of deciduous and permanent teeth	27[d]
rs28933971	g.10408G>C	83G>C	Arg28Pro	Non-syndromic oligodontia	28[d]
None	g.10210G>A+g.10211C>A	128G>A+129C>A	Ser43Lys	Autosom al-dominant molar oligodontia	20 [d]
rs121917720	g.10464C>T	139C>T	Arg47Trp	Autosomal-dominant non-syndromic oligodontia	29[d]
None	g.10465G>C	140G>C	Arg47Pro	Autosomal dominant non-syndromic hypo-/oligodontia	30[d]
None	g.10471C>T	146C>T	Ser49Leu	Sporadic non-syndromic oligodontia	26[d]
rs104894469	g.10476G>A	151G>A	Gly51Ser	Isolated oligodontia, third molars, second premolars and incisors	31[d]
None	g.10477G>C	152G>C	Gly51Ala	Sporadic non-syndromic oligodontia	32
None	g.10492T>C	167T>C	Ile56Thr	Autosomal dominant non-syndromic hypo-/oligodontia	30[d]
None	g.10500ins288bp	175ins288 bp	Arg59Glnins288 bpfsX178	Autosom al-dominant hypodontia	25 [d]
None	g.10500C>T	175C>T	Arg59X	Oligodontia, most molars and second premolars	33
None	g.10544insG	219insG	Gly73fsX243	Autosomal-dominant oligodontia Autosomal-dominant molar agenesis	34,35
None	g.10555_10567del13bp	230_242del13 bp	Arg77_Pro81 delfsX4	Sporadic non-syndromic oligodontia	32
None	g.10563A>G	238A>G	Thr80Ala	Sporadic non-syndromic oligodontia	32
rs104894468	g.10584A>T	259A>T	Ille87Phe	Autosoma l-dominant molar oligodontia	3,6[d]
rs28933373	g.10596A>G	271A>G	Lys91Glu	Autosomal-dominant hypodontia	25[d]
None	g.10650_10651insG	325_326insG	Ala108GlyfsX316	Autosomal-dominant non-syndromic oligodontia	37[d]
rs104894467	g.10665A>T	340A>T	Lys114X	Molar oligodontia Autosomal dominant non-syndromic hypo-/oligodontia	38[d] 30[d]
None	g.10731C>T	406C>T	Gln136X	Autosomal dominant non-syndromic hypo-/oligodontia	30[d]
rs139008563	g.10753A>G	428A>G	Tyr143Cys	Sporadic non-syndromic oligodontia	32
None	g.10758C>T	433C>T	Gln145X	Hypo-/oligodontia	3,9[d]
None	g.10828C>G	503C>G	Ala168Gly	Non-syndromic hypo-/oligodontia	19
rs61734510	g.10841G>A	516G>A	Lys172Lys	Autosomal dominant non-syndromic hypo-/oligodontia	18[d]
None	g.10917_10921duplGTGAC	592_596duplG TGAC	Asp200Serf sX213	Autosoma l-dominant non-syndromic hypo-/oligodontia	1,8[d]

(*Continues*)

TABLE 1 (Continued)

PAX9						
dbSNP rs# cluster id[a]	**Position in whole-gene region[b]**	**CDS/exon position[c]**		**Amino acid change[c]**	**Phenotype**	**Reference, notice**
rs61754301	g.10934C>T	609C>T		Gly203Gly	Hypo-/oligodontia, NSA	6
None	g.10944_10946 del3bpins24bp	615_617del3bpins24bp		Ille207TyrX211 or Ille207TyrfsX315	Autosomal-dominant oligodontia	40[d]
rs12881240	g.13980C>T	717C>T	EXON 4	His239His	Hypo-/oligodontia, NSA Maxillary lateral incisor agenesis (hypodontia), NSA Sporadic non-syndromic oligodontia, NSA Molar agenesis, NSA Sporadic, non-syndromic oligodontia, NSA	41[d] 42[d] 5,17,43
rs4904210	g.13981G>C	718G>C		Ala240Pro (het) Ala240Pro (het/hom) Ala240Pro (hom.) Ala240Pro(het.)	Sporadic tooth agenesis, NSA Sporadic non-syndromic oligodontia, NSA Maxillary lateral incisor agenesis (hypodontia), NSA Hypo-/oligodontia Autosomal-dominant oligodontia—anterior teeth Molar agenesis, crown size, NSA Sporadic non-syndromic hypodontia Non-syndromic oligodontia Sporadic non-syndromic oligodontia Sporadic non-syndromic oligodontia Sporadic non-syndromic anodontia, tooth agenesis, NSA	10,17,42[d] 41[d] 44,45[d] 5,8,43,46,47[d] 48[d]
None			EXON 5	Val264ArgfsX315	Molar oligodontia	49[d]
rs10141087			3´-near gene	intron	Sporadic non-syndromic hypodontia, NSA	8
None				1.176 Mb deletion Non-specific deletion 3.5 Mb deletion 0.9 Mb deletion 0.717 Mb deletion 0.884 Mb deletion 4.08 Mb deletion	Oligodontia Oligodontia Hypodontia Hypodontia Oligodontia Oligodontia Oligodontia	50–56

−, in front of exon (in IVS region); +, behind of exon (in IVS region); del, deletion; fs, frame shift; ins, insertion; NSA, no significant association was found; UTR, untranslated region; X, termination of translation (stop codon) or deletion of AA.

[a] According 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).

[b] According NCBI Reference Sequence: NG_013357.1 (http://www.ncbi.nlm.nih.gov/nuccore/262331554).

[c] According NCBI CCDS Database: CCDS9662.1 (http://www.ncbi.nlm.nih.gov/CCDS/CcdsBrowse.cgi?REQUEST=CCDS&DATA=CCDS9662.1).

[d] Publication with wrong exon counting or wrong location of nucleotide changes.

acid change. Kapadia et al[36] reported similar results for Ille87Phe mutant but detected no additional effects on the interaction between the mutant PAX9 protein and MSX1. The other studies listed in Table 1 show the impact of mutations on PAX9 function only by in silico methods. Most of these studies compare conserved regions of various PAX proteins across animal species.

Suda et al[37] reported g.10650-10651 insertion of guanine causing Ala108Gly substitution and a frameshift mutation resulting in a premature stop codon at amino acid position 316. Authors transfected mutated construct into COS-7 cells to examine functional parameters and found the same level of wild type and g.10650_10651insG mRNA but with significantly higher instability of the mutated mRNA. This probably led to a decreased level of expression of the protein. Other frameshift mutation or mutations leading to a premature appearance of stop codon were, in most cases, published without functional parameters.

Three silent mutations—Gly203Gly, Ile25Ile, Lys172Lys in the third exon have been reported. In our earlier study, we conjectured that Gly203Gly would have no significant impact on the tooth development as it was present in both healthy controls and patients with tooth agenesis.[6] Haddaji Masttouri et al[18] found that Lys172Lys was present only in 4 affected family members and not in 89 control individuals. This might be taken as an indication that the mutation is causative, but an association study with much larger cohort would be needed before more positive conclusions are made. Thimmegowda et al[24] published a case report for the Ille25Ille mutation but it was not entirely clear which family members were/were not the carriers. The precise locus of the mutation was not given and the corresponding protein structure was not included so the putative impact—if there is any—on hypodontia is hard to evaluate.

2.4 | Exon 4

In the exon 4, only 1 synonymous and 1 missense mutations have been found. These have been reported as leading to a variety of phenotypes depending on the size of the cohorts of patients and control subjects and also on the studied population.

None of the 5 authors (Table 1) who identified the synonymous g.13980C>T polymorphism (His239His) could find any cases that would indicate any significant correlations with missing/malformed teeth.

Twelve papers present sometimes contradictory conclusions as to the homozygous and heterozygous genotypes of the g.13981G>C polymorphism which could result in a missense Ala240Pro substitution (rs4904210 polymorphism).

Thus, Pinho et al[42] who studied 12 Portuguese families comprising 52 individuals (12 probands and 40 relatives compared to 91 unrelated controls) found no significant association between maxillary lateral incisor agenesis and rs4904210 polymorphism (67% probands and 56% controls carried the C allele). A Chinese study[10] took a different approach and examined 102 Han Chinese subjects with sporadic tooth agenesis and 116 healthy controls. The rs4904210 polymorphism was not associated with any significant risk of tooth agenesis. In this respect agreeing with Pinho et al[42] but, together with 3 other polymorphisms g.8102A>G, g.13981G>C, g.8158C>G, g.8973C>T, haplotype AGGC may have conferred protection against the risk of sporadic tooth agenesis.

Complicating the matter even further, the rs4904210 polymorphism was the only change found in 1 Chinese Han family represented by 6 individuals with no mutation in *MSX1* gene and *AXIN2* gene leading to a conclusion that Ala240Pro substitution (rs4904210 polymorphism) could be a risk factor for tooth agenesis in Han ethnicity.[45] Wang et al[48] studied sporadic anodontia in Han family consisting of 3 healthy members and a female proband with complete absence of primary and secondary dentitions. They found the rs4904210 polymorphism in the heterozygous state in the female proband and her younger brother and the homozygous genotype CC in her father but not in her mother. This result led to a larger case-control study of the rs4904210 polymorphism including 50 subjects with sporadic tooth agenesis and 100 healthy controls but, again, there was no significant association between tooth agenesis and the polymorphism rs4904210. Liu et al[8] tested 273 subjects diagnosed with non-syndromic hypodontia and 200 healthy control subjects for 25 SNPs in 11 genes potentially involved in odontogenesis. One of these polymorphisms was g.13981G>C (rs4904210). The only positive result for rs4904210 was a weak association of the hypodontia with the allele C in the male group of cases reaching *P*-value of .02. This is not a very high level of statistical significance but it does raise a possibility of an association between rs4904210 and non-syndromic hypodontia, at least in males. More extensive studies perhaps involving linkages with other genetic variations are needed to make more definite conclusions.

Pereira et al[57] suggested that rs4904210 was a neutral polymorphism in humans. They screened 86 healthy individuals from 3 geographical groups (Europeans, Asians and Native Americans) and then they compared them to 3 samples of Polish population—144 healthy subjects, 52 patients who suffered from hypo- or oligodontia and 154 patients with cleft lip palate. The results supported the view that *PAX9* paired domain is strongly conserved across humans and primates while the exon 4 could act as an evolutionary window to functional adaptations in the primate dentition.

In 1 patient with oligodontia was diagnosed homozygous variant CC of rs4904210 polymorphism in a study from Belgium but without any proof of direct impact on teeth formation because of small numbers of patients and control subjects.[47] In another small study,[46] 8 family members of a Mongolian family with non-syndromic oligodontia were G/C heterozygous while the remaining 9 unaffected family members had the standard reference sequence in *PAX9* gene. Thus the probable cause of the oligodontia would seem to be the Ala240Pro change and this would contradict the findings of the Portuguese and Chinese studies discussed earlier.[10,42]

In a study by Paixão-Côrtes et al,[41] 35 “white” Brazilian patients with non-syndromic tooth agenesis including third molars and 15 individuals without any dental agenesis were genotyped for rs4904210 polymorphism. There was no significant statistical difference between 240Pro allele in patients and controls but the analyses indicated possible relationship with a third molar agenesis probably with a recessive pattern of inheritance. The authors suggested mutations out of binding domain of the PAX9 as a possible player in the tooth agenesis. A case report for homozygosity for rs4904210 polymorphism in

all members of 1 family affected with variable tooth agenesis was published by Kula et al.[44] No significant effect on sporadic, non-syndromic oligodontia was found by a Polish study performed on 6 patients by direct sequencing. The allele C rs4904210 polymorphism was detected in only 1 patient.[17]

Lee et al[5] investigated 223 Korean individuals and 273 Japanese subjects with lacking 1 or more molars and genotyped them for several mutations including the rs4904210 polymorphism. There was no significant correlation between the absence of molars and the rs4904210 polymorphism, but the groups displayed significant association between the rs4904210 polymorphisms and crown size. Another interesting outcome of the study was that lacking 1 or more molars in Korean subjects led to larger crown sizes of premolars and molars compared to individuals with all 4 molars.[5]

Zhang et al[58] performed meta-analysis of the data chosen from 4 previous studies related to the rs4904210 polymorphism; Pinho et al,[42] Paixão-Côrtes et al,[41] Liu et al[8] and Wang et al.[48] Their results showed no significant association between hypodontia and the g.13981G>C polymorphism (rs4904210).

2.5 | Exon 5

So far only 1 mutation in exon 5 has been identified by Frazier-Bowers et al.[49] They found an insertion of cytosine at position g.23652 (outside the paired domain). This was present in all affected family members suffering from oligodontia. The mutation would have resulted in a frame shift at amino acid 264, adding 51 nonsense amino acids followed by premature termination of translation at amino acid 315.

2.6 | Intronic sequences

To date, 12 mutations occurring in the intron sequences and near *PAX9* gene regions have been published. The first SNP located before exon 1 at the position g. 4536C>A in the 5′-flanking region was reported as not associated with crown size or third molar agenesis in a study of 2 groups of Japanese and Korean subjects.[5]

The polymorphism rs2073244 (g.8102A>G) in intron 1 was investigated in 2 case-control studies on Han Chinese population; neither of them established any statistical difference between patients and controls.[8,10] Three other studies focusing on the polymorphisms g.8102A>G (rs2073244) and g.8221C>T (rs2073246; also located in intron 1: IVS1) all done on Caucasian subjects (Turkish, Hungarian and Brazilian populations) showed higher risk for hypodontia in patients with mutated GT haplotype. The findings indicated a synergic effect of the 2 polymorphisms. In all 3 studies, these polymorphisms are termed "G-1031A" in the case of rs2073244 and "T-912C" in the case of rs2073246.[9,11,12] There appears to be, however, no specified reference sequence given according to which the polymorphisms were aligned, nor is there any information on the point from where the numbering starts. Thus, the precise position of the apparent polymorphisms is uncertain. In "Background" section of the study by Isman et al[11] "T-912C" change is designated as an SNP with number rs2073247 but that is at variance with the sequence and numbering as it appears in reference sequence NG_013357.1 of the *PAX9* gene (NCBI database) and cannot, therefore, be correct. Furthermore, in their discussion, authors refer to the Chinese study by Pan et al[10] but Pan et al[10] presented results corresponding to the SNP rs2073247 positioned at g.8973C>. Yet Isman et al[11] claimed that they verified rs2073246 as correctly corresponding to another position (g.8221C>T) and cited the NCBI dbSNP Short Genetic Variations database. These contradictory statements are confusing and can easily lead to misinterpretation of important findings. Correct designations of the polymorphisms are given in Table 1.

Bianchi et al[13] reported that G.8158C>G polymorphism in the intron 1 (IVS1) was associated with a specific molar hypodontia. In this study, Caucasian subjects with the CC genotype (C is the non-mutated allele according to the cited database) appeared to be 7 times more probably to develop third molar agenesis than those with the GG genotype. However, it is not clear to which point in the reference sequence was the mutation "G-915C" related; no such polymorphism appears at the designated position in the cited database. The abstract lists the SNP as rs2073247 which is at variance with rs2073245 in the text. Our in silico analysis indicated that the primer sequences specified in the method section correspond to those of rs2073245.

Incidentally, results from Liu et al[8] on the SNP rs2073247 (g.8973C>T; cf. the difference from Bianchi et al[13]) indeed identified the T allele as a risk factor for hypodontia in Chinese Han population. In contrast, Pan et al[10] found no association between rs2073247 and hypodontia. The latter study was also done on Chinese Han population but the size of the sample was smaller, about half of that used by Liu et al[8]; this could explain the apparent discrepancy.

A group of American authors proposed an IVS1 g.8264G>A intron variation as a probably cause of hypodontia in a family study comprising 17 members. Based on bioinformatics data, the variation was localized to a putative 5′-regulatory sequence that is known to be highly conserved in primates. The researchers have been, however, unable to compare the PAX9 mRNA levels between the affected and unaffected individuals mainly because the amounts of mRNA isolated from their source material (lymphocytes and lymphoblasts) were too low to analyse.[14]

More recent Czech study screened 270 subjects with oligodontia and identified a previously unknown heterozygous g.9527G>T mutation located in the *PAX9* gene.[7] This was present in a pair of monozygotic twins and 3 members of their family, all affected by oligodontia. The mutation is located in a conserved region between exon 2 and intron 2 normally accessed by highly specific splicing enzymes in accordance with the so-called GT-AG rule. The mutation violates the rule thus almost certainly disrupting the splicing process and having major consequences for the availability of the normal PAX9 protein.[7]

Three other mutations in the intron 2 (IVS2) were examined for their possible relationship with tooth agenesis. Two studies including one our study investigating population samples from Central European region (Czech Republic and Poland) and the other 2 were Tunisian and Brazilian family studies. Polymorphisms rs12883298, rs12882923 and rs12883049 were identified as having possible influence on the occurrence of hypodontia but all 4 studies recommended further research with larger cohorts of subjects.[6,17–19]

Liu et al[8] studied a polymorphism with a designation rs10141087 (g.25569G>C) located in an intron region near the 3′-end and reported no association with the non-syndromic hypodontia.

2.7 | Whole PAX9 gene deletions

A special type of spontaneous genetic modification is a deletion of long section of the chromosome 14 including whole *PAX9* gene located on 14q13.3. As the deletions include also other genes in the vicinity, the literature extends over a range of defects caused by severe disruptions of the developmental process and affecting also organs other than teeth. It is, therefore, no surprise that, in this context, the hypodontia and oligodontia have not always been at the forefront of the main research interests and only a few relevant papers have appeared (See Table 1). The publications discussing the deletions of the PAX9 gene do so usually in combination with the deletions of *FOXG1*, *GARNL1*, *NKX1-2*, *TTF1* (depending on the length and location of the missing DNA). In fact, all known affected individuals suffered from tooth agenesis and this is in accordance with the haploinsufficiency of *PAX9* gene being a high-risk factor for hypodontia or oligodontia in humans.[54,59]

3 | CONCLUSIONS AND THEORIES

Mutations in various regions of PAX9 gene lead to diverse outcomes. For example, mutation in a coding sequence of the *PAX9* gene may lead to a loss of function if it adversely impacts on characteristics (such as binding affinity) of a DNA-binding domain specific for a transcription factor (eg, MSX1). It may also alter its nuclear localization. The haploinsufficiency of the PAX9, both in vivo and in vitro is a proven cause of tooth agenesis. It may happen by a variety of mechanism, for example, by damage to the initiation codon, mutations resulting in a frameshift and leading to a premature stop codon or deletions of long sections of the chromosome. Some mutations can even give rise to a novel protein with an unknown function.

The same mutation or the same impact on coding or non-coding elements of the *PAX9* gene can lead to diverse outcomes in the phenotype. These observations evince very high-genetic heterogeneity of *PAX9*. Obviously, all growth and transcriptional factors have to be in the right place, at the right time and in an exact quantity at every stage of the ontogenetic development. Critical involvement of a complex gene such as PAX9 in odontogenesis presents ample opportunities for a variety of mutations to disturb the fragile balance.

The nucleotide change can occur also in a region of the *PAX9* gene which does not significantly affect the protein structure or its function but it may have influence on the rate of production of the protein (and ultimately the quantity produced) by altering transcription, accessibility of genetic components, transport and possibly much more.

We cannot ignore the influence of the untranslated regions on the expression of the protein because of the possible presence of regulation elements as well as binding sites for miRNAs molecules. It is known that small non-coding RNAs molecules (miRNAs) affect expression of genes and signalling network between epithelium and underlying mesenchyme. No data directly implicating *PAX9* gene in this process have appeared to date *PAX9* but Jevnaker and Osmundsen[60] detected about 76 miRNAs at E15.5, P0 and P5 in mouse first molar's tooth buds which could potentially induce the developmental timing through fine-tuning of mRNA targets. In another recent development, it has been showed by Ma et al[61] in vitro and in vivo that miR-3649 could interacts with rs12532 in a non-coding region of the *MSX1* gene and this may have implications for the origin of non-syndromic orofacial clefts. The presence of a specific genotype that carries a binding site for miR-3649 correlates with the expression of the defect.[61]

Non-nucleotide changes known as epigenetic alterations such as DNA methylation and histone modification, could also play a role in the mechanisms leading to oligodontia. Pilot study of the impact of DNA methylation as a part of epigenetic modification revealed differences in the methylation status of several genes (6636) between individuals with non-syndromic tooth agenesis and controls. The 9 genes with highest differences in methylation status have undergone further research including NFκBIB, PRKCD, Hey1 CACNA1A. All these genes are related to cell signalling pathways important during tissue and organ development.[62]

Many of the reviewed articles suffered from incorrect numbering of exons of the *PAX9* gene (in Table 1 referred with footnote 4). This could have been caused by using old reference sequences or by counting only the exons containing the coding regions. However, as we discussed above, mutations outside the protein-coding region may also play an important role in gene expression or its regulation. The problem is further compounded by erroneous location of mutations and missing reference sequences in some articles thus making potentially important studies hard or impossible to evaluate. It should be a requirement to specify how the numbering of a mutation was made, use a standard international nomenclature and cite the accession numbers of the reference sequence obtained from National Center for Biotechnology Information (NCBI) database. Ideally, the location should be given using all routinely available types of referencing, for example, the rs number of single nucleotide polymorphism (SNP), gene reference sequence, mRNA reference sequence and coding sequence (CDS) reference sequence to minimise potentially confusing information.

It should be clear from the above discussion that family studies, while providing a very good model for identifying new relationships between mutations and absence of teeth, cannot explain the precise nature of the associations between polymorphisms and tooth agenesis.

Association studies have a potential to produce discordant outcomes because each has its own set of limiting parameters—type of population, the number of studied individuals, the number of comparable control samples interrogated for comparison, great variety of phenotypes (32 unique teeth), specificity of the impact of the mutation on primary and/or secondary teeth, use of different methods for statistical analysis, the large number of genes involved in tooth development which can harbour additional mutations and, also, different impact of mutations in different populations.

There is a paucity of detailed studies of the functions, or, at least interactions of the mutated proteins. If anything, the trend shows a shift towards theoretical comparisons of "normal" and mutated proteins using bioinformatics and in silico approach. At the same time, there are few options available to test physiological levels of PAX9 because of its extremely low expression in adult humans.

Most of the reviewed studies used capillary sequencing or restriction enzyme analysis of polymerase chain reaction products as

the genotyping approach. These methods are suitable for studying specific SNP's or small selected fragments of DNA (eg, single exons). What is needed, however, is a rapid access to much larger amounts of relevant genetic information such as whole-gene analyses performed on a particular population sample. The next-generation sequencing is capable of bringing such outcomes and could play an important part in the association studies in coming years. Larger amounts of data would not only help to identify many new mutations, but enable to compute combinations of polymorphisms in multiple genes and reveal synergistic/protective effects of SNPs which may play very important roles in tooth development.

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Conflict of interest

The authors declare no conflict of interest related to this work.

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Příloha II – Mutations in *AXIN2* gene as a risk factor for tooth agenesis and cancer: A review

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REVIEW ARTICLE

Mutations in *AXIN2* gene as a risk factor for tooth agenesis and cancer: A review

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Abstract

AXIN2 gene plays a crucial role in morphogenesis of craniofacial area and is essential for tooth development. *AXIN2* gene is one of the most studied genes associated with tooth agenesis, the most common defect of dentition in humans. Polymorphic variants in *AXIN2* gene are discussed in relation to the occurrence of the tooth agenesis but also as an indication of the risk of cancer. Mutations in *AXIN2* gene were found in patients with colorectal or hepatocellular carcinoma, prostate cancer, ovarium or lung cancer. These findings support the hypothesis that missing teeth may be a significant marker for predisposition for cancer.

Abbreviations:

APC	- adenomatous polyposis coli
AXIN2	- axis inhibitor 2
βTrCP	- β-transducin repeat-containing protein
CDS	- coding sequence
CK1	- casein kinase 1
dbSNP	- single nucleotide polymorphism database
DIX	- Dishevelled/Axin domain
Dvl	- Dishevelled segment polarity proteins
EDA	- ectodysplasin A
EDAR	- ectodysplasin A receptor
EDARADD	- EDAR associated death domain
Fzd	- G protein-coupled receptors, class F frizzled
GSK-3β	- glycogen synthase kinase 3β
kbp	- kilo base pairs
KRT17	- keratin 17
LEF	- lymphoid-enhancer factor
LRP	- low density lipoprotein
LRP6	- low-density lipoprotein receptor related protein
miRNA	- micro RNA
mRNA	- messenger RNA
MSX1	- muscle segment homeobox 1
NEMO	- NF-κB essential modulator
PAX9	- paired box 9
RGS domain	- regulator of G protein signaling domain
RNA	- ribonucleic acid
SNP	- single nucleotide polymorphism
SNPs	- single nucleotide polymorphisms
TCF	- T-cell factor
UTR	- untranslated region
Wnt	- wingless-type MMTV integration site family
WNT10a	- wingless-type MMTV integration site family member 10a

INTRODUCTION

Tooth agenesis is defined as a condition where one or more teeth are missing because of a developmental failure. It is the most frequent craniofacial defect in humans (Matalová *et al.* 2008). Over 20 % of world population have missing third molars (Carter & Worthington 2015) and, even if we exclude the absence of the third molars, up to 10 % of the human population is affected by agenesis of permament teeth of one kind or another (Vastardis 2000; Mattheeuws *et al.* 2004). The prevalence of tooth agenesis varies geographically and is influenced by ethnic background (Polder *et al.* 2014). Another consequence of tooth agenesis is the presence of structural defects in the oral cavity leading to functional complications (Stevenson *et al.* 2015). The affected individuals may have difficulties with phonetic articulation and chewing (Koussoulakou *et al.* 2009).

Tooth agenesis is classified as a clinically and genetically heterogenic condition (Kapadia *et al.* 2006) affecting preferentially certain groups of teeth such as the third molars, second incisors and second premolars (Stevenson *et al.* 2015). The condition may exist as an isolated non-sydromic variation or as a part of a broader syndrome where, in addition to the missing teeth, other tissues and organs are affected (Kapadia *et al.* 2006). Multiple tooth agenesis may be associated with specific syndromes or abnormalities (Krejčí 2006). Like majority of craniofacial defects the tooth agenesis is thought to be a multifactorial disorder (Švábová *et al.* 2012; Rakhshan 2015; Stevenson *et al.* 2015; Azzaldeen *et al.* 2017, Bonczek *et al.* 2017). Etiology of the tooth agenesis is, therefore, likely to be very complex and may involve both genetic and enviromental factors (Mostowska *et al.* 2006).

Tooth development is complex multistep process involving a dynamic array of many interacting molecules; their most likely role being to effectuate and/or facilitate the continuous communication between epithelial cells of the developing oral cavity and adjacent mesenchymal cells. The relevant genes have to be expressed in a definite spatiotemporal manner and

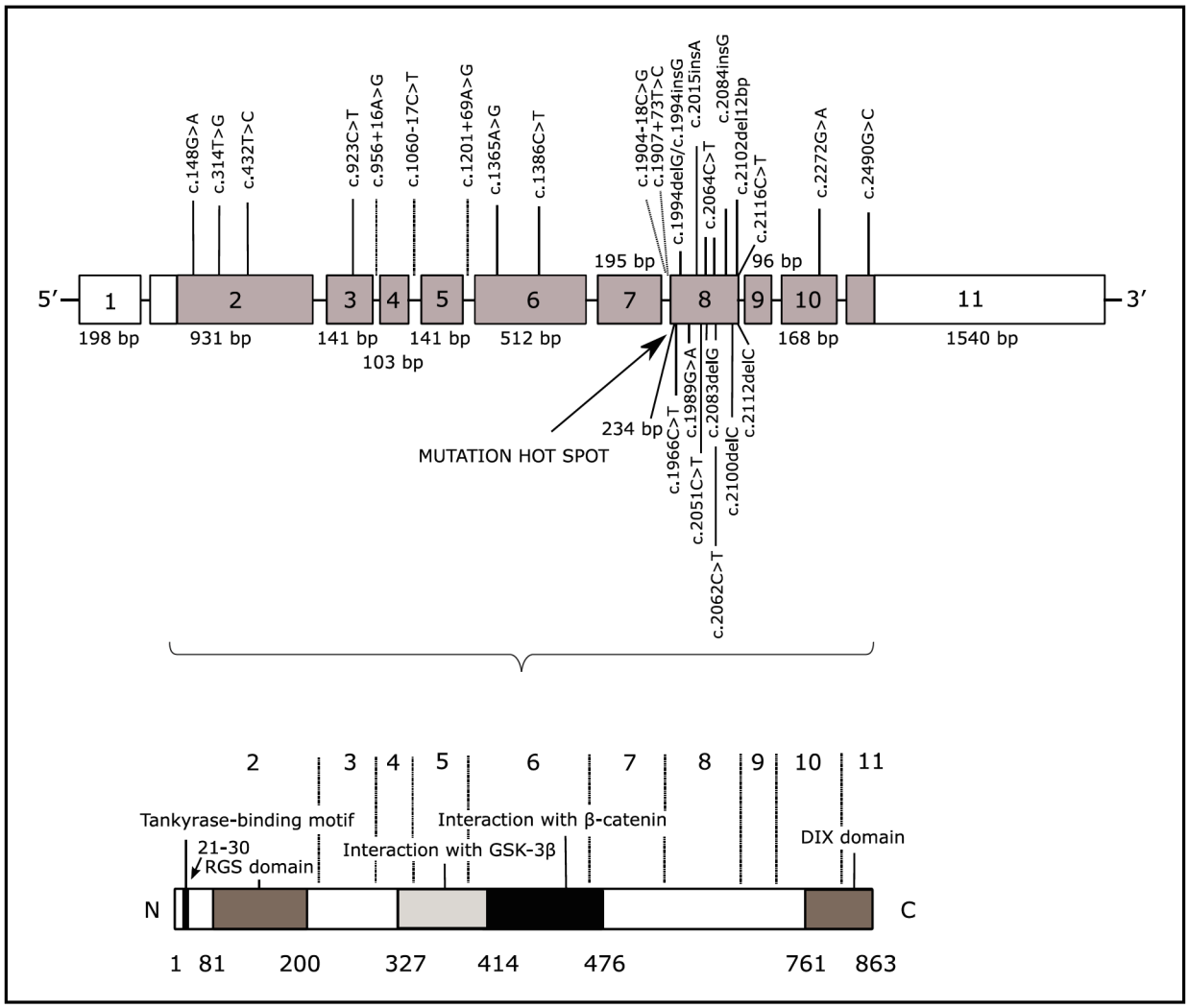

Fig. 1. Schematic description of nucleotide sequence and protein structure of the AXIN2 gene. Light grey colour shows translated region (UTR).

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form a complex molecular network capable of exerting precise and delicate control over the process of odontogenesis (Lin *et al.* 2007). More than 300 genes are known to be involved in odontogenesis (Wright 2015; Al-Ani *et al.* 2017), and mutations in several of these, specifically in *PAX9, MSX1, AXIN2, WNT10a, EDA, EDARADD, NEMO* and *KRT17* genes, have been associated with tooth ageneses (Ruf *et al.* 2013; Šerý *et al.* 2015; Hloušková *et al.* 2015; Bonczek *et al.* 2017).

One of the most studied genes related to tooth agenesis is the gene *AXIN2*. *AXIN2*, or, in particular, the protein which it expresses (axis inhibitor protein 2) plays an important role in the morphogenesis of craniofacial region (Han *et al.* 2014). *AXIN2* gene is expressed during morphogenesis in the dental mesenchyme surrounding the epithelium in the upper and lower jaw and also in the enamel knot, in the mesenchyme of dental papilla as well as in the layer of mesenchymal odontoblasts and preodontoblasts (Lammi *et al.* 2004). Patients with polymorphic variants of *AXIN2* gene have missing permament molars, premolars, lower incisors and upper side incisors (Matalová *et al.* 2008). In addition to causing the tooth agenesis, the mutations in *AXIN2* gene may be associated with carcinogenesis. Mutations in *AXIN2* gene have been found in patients with colorectal or hepatocellular carcinoma and in lung, prostate and ovarian cancers (Lammi *et al.* 2004; Salahshor & Woodgett 2005; Ma *et al.* 2014).

AXIN2 GENE STRUCTURE

AXIN2 gene comprises 4241 bp and is located on the long arm of chromosome 17 in region 17q24.1. The gene consists of 11 exons from which 10 are coding exons and the whole coding sequence is more than 2.5 kbp long (Kanzaki *et al.* 2006). Exon 1 is not translated and, therefore, represents a 5′-untranslated region (UTR). A part of exon 2 also includes a 5′-UTR. The remaining exons are translated into protein stucture. A part of exon 11 is a 3′-UTR (http://www.ncbi.nlm.nih.gov/nuccore/NM_004655). Some authors have not been taking into account mutations in exon 1 as having effects on the function of Axin2 protein (Lammi *et al.* 2004; Marvin *et al.* 2009; Mostowska *et al.* 2006; Taniguchi *et al.* 2002; Gunes *et al.* 2009). However, as we have noted in our recent review, polymorphisms in the untranslated regions should not be ignored because of possible presence of sites for transcription factors and sequences targeted by miRNAs molecules (Bonczek *et al.* 2017).

PROTEIN AXIN2 AND ITS ROLE IN WNT SIGNALING PATHWAY

AXIN2 gene encoded protein Axin2 is composed of 843 amino acids (Salahshor & Woodgett 2005). Axin2 is a part of multiprotein complex taking part in regulating the canonical Wnt signaling pathway (Jho *et al.* 2002). This pathway directs many cell processes in organism. It plays a crucial role in ontogeny where it controls cell proliferation and differentiation (MacDonald *et al.* 2009) but it is also important for homeostatic self-renewal in a number of adult tissues (Clevers 2006). Wnt signaling pathway is closely connected with the regulation of life cycle such as the division of stem cells and their regeneration. Constitutive activation of the pathway leads to the development of neoplasia and tumors, particularly those of colorectum, skin, stomach, lungs, kidneys, prostate, ovaries and breasts (Clevers & Nusse 2012; Dong *et al.* 2001; Alanazi *et al.* 2013).

The canonical Wnt signaling pathway is regulated by an extracelular ligand (Wnt) interacting with the transmembrane receptor Frizzled (Fzd); (Clevers & Nusse 2012). The presence of seven transmembrane hydrophobic domains suggests that Fzd is a G protein-coupled receptor. The absence of extracelular ligand Wnt leads to phosphorylation of β-catenin multiprotein degradation complex consisting of Axin2, tumor supresor APC (adenomatous polyposis coli), CK1 (casein kinase 1) and GSK-3β (glycogen synthase kinase 3β). Phosphorylated β-catenin is recognized by βTrCP (β-transducin repeat-containing protein), E3 subunit of ubiquitin ligase and is thereafter rapidly degraded in proteasome resulting in its low cytoplasmic level (MacDonald *et al.* 2009).

In contrast, coupling of the Wnt ligand to Fzd leads to an interaction between Fzd and a coreceptor LRP6 (or LRP5; low-density lipoprotein receptor related protein). Following the translocation of a protein Dvl towards the cytoplasmic domain of Fzd, the proteins polymerize. Thus organised Dvl clusters interact with Axin2 which is shifted to the intracellular domain of LRP in the process. In addition to Axin2, kinases GSK-3β and CK1 are also translocated at the same time and in the same direction resulting in LRP phosphorylation and subsequent inactivation of the degradation complex. As the complex breaks down, β-catenin is not phosphorylated and is, instead, accumulated in the cytoplasm. The accumulated protein eventually enters the nucleus creating a complex with the transcription family TCF (T-cell factor)/LEF (lymphoid-enhancer factor) while stimulating expression of Wnt target genes (Dong *et al.* 2001; Salahshor & Woodgett 2005; MacDonald *et al.* 2009; Clevers & Nusse 2012).

AXIN2 DOMAINS REQUIRED FOR INTERACTION WITH ITS PARTNERS IN MULTIPROTEIN DEGRADATION COMPLEX

The scaffold formed by Axin2 molecules in the multiprotein degradation complex interacts with other members of the complex (Jho *et al.* 2002; Dong *et al.* 2001) and, in this manner; Axin2 can consecutively coordinate the phosphorylation of β-catenin (MacDonald *et al.* 2009). Axin2 contains two highly conserved

domains. The first domain is designated RGS (regulator of G protein signaling, aa 81-200) and includes a binding site for APC while the second domain designated as DIX (Dishevelled/Axin, aa 761-843) contains a binding site for Dvl protein. Axin2 futher contains sites for interaction with, respectively, GSK-3β (aa 327-413) and β-catenin (aa 413-476); (Salahshor & Woodgett 2005). Apart form the sequences binding other members of the multiprotein complex Axin2 contains a motif for interaction with tankyrase (aa 21-30). Tankyrase stimulates degradation complex trough poly-ADP-ribosylation by the ubiquitin proteasome system (Huang *et al.* 2009).

AXIN2 GENE VARIANTS AND THEIR LINKAGE WITH TOOTH AGENESIS AND CANCER

To date, several SNPs and mutations influencing Axin2 function have been identified. They relate not only to the tooth agenesis but also to a number of tumors in various tissues and organs (Table 1).

Nonsense and frame shift variants

Lammi *et al.* (2004) described four generations of a family with an inherited missense mutation c.1966C>T

Tab. 1. Summary of mutations in the *AXIN2* gene.

dbSNP rs# id (NCBI)	CDS position	localization	amino acid change	variant	phenotype	reference
rs2240308	c.148G>A	exon 2	p.Pro50Ser	missense	lung cancer prostate cancer tooth agenesis	Kanzaki *et al.* (2006); Gunes *et al.* (2009); Ma *et al.* (2014); Callahan *et al.* (2009); Mu *et al.* (2013)
rs752881223	c.314T>G	exon 2	p.Val105Ala	missense	nonsyndromic oligodontia	Liu *et al.* (2015)
rs2240307	c.432T>C	exon 2	p.Ile144	silent	cleft palate	Han *et al.* (2014)
none	c.923C>T	exon 3	p.Thr308Met	missense	nonsyndromic oligodontia	Wong *et al.* (2014)
none	c.956+16A>G	intron 3	–	intronic	tooth agenesis	Mostowska *et al.* (2006)
none	c.1060-17C>T	intron 4	–	intronic	nonsyndromic oligodontia	Mostowska *et al.* (2006); Mu *et al.* (2013)
none	c.1201+69A>G	intron 5	–	intronic	nonsyndromic oligodontia	Mu *et al.* (2013)
rs9915936	c.1365A>G	exon 6	p.Pro455	silent	nonsyndromic oligodontia	Mu *et al.* (2013)
rs1133683	c.1386C>T	exon 6	p.Pro462	silent	nonsyndromic oligodontia astrocytosis	Mu *et al.* (2013); Gunes *et al.* (2010)
none	c.1904-18C>G	intron 7	–	intronic	nonsyndromic oligodontia	Mu *et al.* (2013)
none	c.1907+73T>C	intron 7	–	intronic	nonsyndromic oligodontia	Mu *et al.* (2013)
rs121908568	c.1966C>T	exon 8	p.Arg656Ter	nonsense	oligodontia, colorectal carcinoma	Lammi *et al.* (2004)
rs730882193	c.1989G>A	exon 8	p.Trp663Ter	nonsense	oligodontia, colorectal carcinoma	Marvin *et al.* (2011)
rs267606674	c.1994delG	exon 8	p.Gly665AIafs	frameshift	colorectal carcinoma	Liu *et al.* (2000)
none	c.1994insG	exon 8	p.Glu706Ter	frameshift	oligodontia	Lammi *et al.* (2004)
none	c.2015insA	exon 8	p.Glu706Ter	frameshift	colorectal carcinoma	Liu *et al.* (2000)
rs138287857	c.2051C>T	exon 8	p.Ala684Val	missense	nonsyndromic agenesis	Bergendal *et al.* (2011)
none	c.2062C>T	exon 8	p.Leu688	silent	tooth agenesis	Mostowska *et al.* (2006)
none	c.2064C>T	exon 8	p.Arg659Trp	missense	hepatocellular carcinoma	Taniguchi *et al.* (2002)
none	c.2083delG	exon 8	p.Leu688Ter	frameshift	colorectal carcinoma	Liu *et al.* (2000)
none	c.2084insG	exon 8	p.Glu706Ter	frameshift	colorectal carcinoma	Liu *et al.* (2000)
none	c.2100delC	exon 8	p.Leu688Ter	frameshift	colorectal carcinoma	Liu *et al.* (2000)
none	c.2102del12bp	exon 8	p.delThrThrProArg	frameshift	hepatocellular carcinoma	Taniguchi *et al.* (2002)
none	c.2112delC	exon 8	p.Leu688Ter	frameshift	colorectal carcinoma	Liu *et al.* (2000)
rs121908567	c.2116C>T	exon 8	p.Glu706Ter	nonsense	colorectal carcinoma	Liu *et al.* (2000)
rs145007501	c.2272G>A	exon 10	p.Ala758Thr	missense	oligodontia, colorectal carcinoma	Bergendal *et al.* (2011)
none	c.2490G>C	exon 11	p.Met830Ile	missense	nonsyndromic oligodontia	Wong *et al.* (2014)

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(p.Arg656Ter) associated with syndromic oligodontia (missing of six and more teeth) and colorectal carcinoma. Eleven family members were missing at least eight teeth; two of them had only three permanent teeth. Most of the family members had normal deciduous teeth. Various types of colorectal carcinoma or precancerous lesions were present in eight family members who also had oligodontia. Lammi *et al.* (2004) also reported heterozygous 1-bp insertion in nucleotide position c.1994insG (nonsense mutation) in a 13-year-old boy with a phenotype very similar to that of the family with the mutation c.1966C>T. The reported insertion creates a new stop codon (Glu706Ter). There was no confirmed cancer in this case but because of the age (13 years) of the proband it might not yet have developed.

Marvin *et al.* (2009) reported four members of a three generation family carrying a nonsense mutation c.1989G>A (p.Tyr663Ter) creating an early stop codon. The phenotype was an autosomal dominant oligodontia variably accompanied by other pathological findings such as intestinal and stomach polyps, mild ectodermal dysplasia and low hair and eybrows density. The proband, her mother, two aunts as well as her deceased maternal grandmother, all had oligodontia. The latter died at 97 though and neither the intestinal polyps nor the bowel cancer were known to be present at the time her death.

Liu *et al.* (2000) identified three 1-bp deletions c.2083delG, c.2100delC and c.2112delC (all p.Leu688Ter) and two 1-bp insertions c.2084insG and c.2015insA (both p.Glu706Ter) in 11 individuals in a group of 45 patients suffering from colorectal carcinoma. Insertions c.2084insG and c.2015insA generate an early stop codon. The result of those frameshift mutations would stabilize β-catenine and to activate β-catenine/T-cell factor signalling. Excluding one patient with the variant c.2100delC, in all remaining 10 individuals Liu *et al.* observed accumulation of β-catenine in the nuclei of the tumour cells by immunocytochemistry using β-catenine antibodies. All these five variants are located in exon 8. In the same exon Taniguchi *et al.* (2002) identified missense mutation c.2064C>T (p.Arg659Trp) and 12-bp deletion starting at position 2102 (c.2102delThrThrProArg) in two of 73 patients with hepatocellular carcinoma.

Missense variants

Kanzaki *et al.* (2006) found an association of c.148G>A mutation (p.Pro50Ser) with lung cancer. This relationship was confirmed by Gunes *et al.* (2009) who had studied 100 patients with lung cancer from Turkey. Significant association between c.148G>A mutation and prostate cancer was established (Ma *et al.* 2014) but no significant associations were reported for the ovarian cancer (Mostowska *et al.* 2014), astrocytoma (Gunes *et al.* 2010), colorectal carcinoma or head and neck tumors (Kanzaki *et al.* 2006). Callahan *et al.* (2009) found a significant association between c.148G>A and tooth agenesis in a group of 116 patients from Brazil who had at least one missing incisor. Presence of a missense variant c.148G>A (p.Pro50Ser) in a group of Mexican patients with isolated nonsyndromatic oligodontia was also noted by Mu *et al.* (2013).

Liu *et al.* (2015) discovered missense mutation c.314T>G (p.Val105Ala) in a Chinese boy suffering from tooth agenesis. This substitution is found in the evolutionary conserved RGS domain of protein Axin2 containing a binding site for APC. The mutation manifested as a non-syndromic oligodontia. Because of the young age of the proband the cancer may not yet have developed so the association with the mutation is uncertain.

In three probands with non-syndromic oligodontia who were missing from seven to ten teeth, Bergendal *et al.* (2011) identified two new missense mutations c.2272G>A (p.Ala758Thr) and c.2051C>T (p.Ala684Val). None of the patients or relatives showed signs of colorectal carcinoma. One of the patients, a 17-year-old girl and her mother were carrying the same frameshift mutation c.1994insG which has been described previously by Lammi *et al.* (2004) in a 13-year-old boy with oligodontia.

In addition to the frame shift variant in exon 8 (c.2102delThrThrProArg) mentioned above, Taniguchi *et al.* (2002) reported a missense mutation c.2064C>T (p.Arg659Trp) in two probands with hepatocelular carcinoma.

Wong *et al.* (2014) studied all ten coding exons of the *AXIN2* gene in Chinese population. In a group of 96 nonrelated patients with non-syndromic oligodontia they identified two new missense mutations. The mutation c.923C>T (p.Thr308Met) was found in a 12-year-old girl who was missing 12 permament teeth (five incisors and seven premolars). Another missense mutation, c.2490G>C (p.Met830Ile), was found in a 22-year-old male with agenesis of twelve teeth (all second premolars, all upper incisors and two lower incisors). Both mutations arose *de novo*. There was no tooth agenesis or cancer in any members of the two probands' families.

Silent and intronic variants

Han *et al.* (2014) examined relationships between four polymorhisms (rs2240307, rs11867417, rs2240308, and rs7591) and the occurrence of nonsyndromic orofacial cleft in Chinese population. The group consisted of three subgroups: patients with, respectively, cleft lip, cleft palate and both. Results indicated association between rs2240307 c.432T>C (p.Ile144) and the presence of cleft palate.

Mostowska *et al.* (2006) were searching for variants in *AXIN2* in a group of 55 patients with tooth agenesis. None of them had any mutations in MSX1 (muscle segment homeobox 1) or PAX9 (paired box 9), i.e. in two genes that are among the most thoroughly studied ones

in relation to tooth agenesis. They found 29 patients with hypodontia (missing less than six teeth) and 26 patients with oligodontia. Additionally, they identified two intronic variants c.956+16A>G, c.1060-17C>T and one synonymous SNP c.2062C>T (p.Leu688) in exon 8.

Flanking regions of exon-intron boundaries were studied by Mu *et al.* (2013) in two Mexican families with isolated non-syndromic oligodontia. Their findings included not only the variant c.148G>A (p.Pro50Ser) mentioned above, but also the presence of two silent variants c.1365A>G (p.Pro455) and c.1386C>T (p.Pro462) and four SNPs c.1060-17C>T, c.1201+69A>G, c.1907+73T>C, c.1904-18C>G in intronic regions. These intronic SNPs may have influenced RNA splicing and lead to changes in cleavage positions for restriction enzymes. Variant c.1386C>T (p.Pro462) was associated with astrocytoma by Gunes *et al.* (2010) in Turkish population.

DISCUSSION

Nonsense mutations and mutations resulting in a shift of open reading frame lead to occurence of premature stop codons leading to an earlier termination of the translation. Considering that these mutations are all concentrated in exon 8 and the premature stop codons arise in positions 656 (Lammi *et al.* 2004), 663 (Marvin *et al.* 2011), 688 (Liu *et al.* 2000) or 706 (Lammi *et al.* 2004; Liu *et al.* 2000), the corresponding variant protein ends up shorter by three exons. This causes the loss of DIX domain on aa 761-843 that is necessary for interaction with Dvl thus leading to disruption of Wnt signaling pathway.

Missense mutations change the amino acid sequence but the final protein has the same length. However, in some cases, e.g. when the amino acid swap results in a change in polarity (i.e. a neutral residue is exchanged for a charged one or vice versa) the final conformation and function of Axin2 may be altered.

Silent mutations should not have a significant influence on protein structure and activity because the amino acid sequence remains the same regardless of the base change. However, it has been observed that, in some cases, RNA splicing may be adversely affected, specific binding sites for exon splicing enhancers may change and, occasionally, binding sites for restriction enzymes could change, too (Mu *et al.* 2013; Mostowska *et al.* 2006).

Most of the mutations and SNPs of *AXIN2* gene are clustered in coding sequence of the 8th exon. There are a number of mononucleotide repetitions between codons 636-714 which could be prone to mutagenesis.

Little attention has been given to intronic polymorphisms because introns are spliced and eliminated in trascription. Recent findings, however, suggest that polymorphisms within introns may have an impact on mRNA splicing (Sweet & Michaelis 2011; Šerý *et al.* 2013; Caminski *et al.* 2014).

Mutations or SNPs in *AXIN2* gene may lead not only to tooth agenesis but also to development of cancer in various organs later in life. Therefore, dentists should be aware of possible relationship between tooth agenesis and, whenever possible, use this knowledge to help to detect carcinoma in its early stages.

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Příloha III – Využití moderních DNA metod ve výzkumu molekulárních příčin hypodoncie

Využití moderních DNA metod ve výzkumu molekulárních příčin hypodoncie

Modern DNA methods in the research of molecular causes of hypodontia

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Souhrn

Příčinou ageneze zubů jsou poruchy buněčné diferenciace v průběhu vývoje dentice.Do současné doby byly nalezeny desítky mutací zasahujících do exprese genů, které se podílejí na diferenciaci buněk dentice. Jedná se především o mutace genů pro PAX9, MSX1 a AXIN2. Výzkum vlivu mutací genů podílejících se na vývoji dentice je založený na sekvenování především translatovaných oblastí. Do současnosti jsou používány běžné kapilární sekvenátory I. generace. Jejich použití je limitováno cenově a pracností analýz. Poslední roky přinášejí nové sekvenační metody (II. generace), zlevnění analýz a pokrytí větších analyzovaných úseků DNA.

Cílem tohoto článku je shrnutí poznatků o dosavadním způsobu výzkumu vlivu mutací na vývoj dentice (Ortodoncie 2013, 22, č. 3, s. 173-178).

A***bstract***

Disturbances of cell differentiation in dentition development are cause of tooth agenesis. Until now, tens of mutations involved in gene expression related to dentition development were found. They are mutations mainly in PAX9, MSX1 and AXIN2 genes. The research on the influence of gene mutations involved in the dentition development is based on the DNA sequencing of primarily translated sequences. Capillary sequencing instruments of first generation were mainly used in the research until now. Their use is limited by cost and by laboriousness. Last years bring new sequencing methods (II. generation) with cheaper analyses and possibilities to make sequencing of larger DNA segments.

The aim of this paper is a summary of the current knowledge about research manner of influence of gene mutations on dentition development (Ortodoncie 2013, 22, No. 3, p. 173-178).

Klíčová slova: *mutace, polymorfizmus, gen, PAX9, MSX1, DNA, oligodoncie, hypodoncie, ageneze zubů*
Key words: *mutation, polymorphism, gene, PAX9, MSX1, DNA, oligodontia, hypodontia, tooth agenesis*

Úvod

Ageneze zubů je běžnou vývojovou poruchou člověka, která postihuje okolo 20% populace. Nejčastěji chybějícím zubem je třetí molár, jeho ageneze v české populaci

Introduction

Tooth agenesis is common developmental disorder of humans that affect about 20% of population. Third molar is most commonly missing tooth, his agenesis

je 22,5% [1]. Prevalence ageneze dalších stálých zubů v obecné populaci kolísá od 2,6% do 11,3% [2].Pro popis numerických anomálií zubů se v literatuře používá několik termínů. Nejstarší z nich, oligodoncie, v pravém slova smyslu znamená „několik zubů“ [3] a v současnosti se doporučuje používat jej pro případy, kdy chybí kromě třetích molárů více než šest zubů. Pro oligodoncii se prevalence v populaci odhaduje na 0,14 - 0,25 %.

Obecně používaný výraz „vrozeně chybějící zuby“ je nepřesné označení pro stálé zuby, které nejčastěji chybí. Tento stav lépe vyjadřuje termín ageneze, který zahrnuje i související vývojové defekty [4].

Hypodoncie je chápána jako komplexní jednotka zahrnující v sobě veškeré anomálie počtu zubů a dále také související anomálie tvaru, velikosti, vývoje a doby erupce zbývajících zubů. V užším slova smyslu se za hypodoncii považují stavy, kdy chybí vyjma třetích molárů do šesti zubů [5].

Ageneze zubů může být součástí syndromů nebo je non-syndromická. Pro výzkum hypodoncie je podstatné její dělení na sporadickou a familiární. Familiární ageneze zubů může být dědičná autozomálně dominantně, autozomálně recesivně nebo může být vázána na X chromozóm. Její výskyt je pozorován také u dalších příbuzných. Uvažuje se, že za příčinou sporadické formy hypodoncie stojí multifaktoriální podmíněnost - vliv prostředí a kombinace vlivu více genů. Mezi faktory prostředí, které mohou ovlivňovat patogenezi hypodoncie patří infekce (např. zarděnky), některé chemikálie a léky, radioterapie, fraktury atd.

Ageneze zubů vzniká jako následek poruchy epitelio-mezenchymálních interakcí, na nichž se podílí mnohé signální dráhy. Předpokládá se účast více než 350 signálních molekul.

Výzkum odontogeneze

Současný výzkum odontogeneze je založen především na myších modelech. Na myších je zkoumán proces vývoje zubů - zapojování jednotlivých signálních molekul v daném místě a čase. Provádějí se histologické řezy dentice v různých vývojových stádiích. Histologické preparáty je možné barvit značenými protilátkami. Používají se speciální protilátky, které jsou značeny fluorescenčními barvami a jsou viditelné při použití fluorescenčního mikroskopu. Samotné protilátky bývají specifické pro detekci jednotlivých signálních molekul. Je tak možné zviditelnit tu část histologického preparátu vyvíjející se dentice, kde právě probíhá signální dráha využívající danou signální molekulu. Pokud se preparáty připraví v různých časech vývoje dentice, je možné sledovat postupné zapojování signálních molekul v jednotlivých vývojových krocích.

Dále se používají transgenní myši (tzv. knock-out), tj. laboratorně upravené myši, u kterých byl z funkce

is in Czech population 22,5% [1]. The prevalence of other tooth agenesis in population vary between 2,6% and 11,3% [2].

Several terms for a description of numerical anomalies of the teeth are used in literature. The most old of them, oligodontia, means „few teeth“[3] and it is recently recomended to use this term when more than 6 teeth are missed with exception of third molars. Prevalence of oligodontia in population is estimated at 0,14 - 0,25 %.

Generally used term „congenitally missed teeth“ is inaccurate term for permanent teeth that are most often missed. The term agenesis with related developmental defect better define this situation [4].

Hypodontia is understood as a comprehensive unit including all the anomalies of the teeth numbers and also related anomalies of shape, size, development and the time of eruption of remaining teeth. In narrower sense, hypodontia is considered as no more than six teeth are missed; expect third molars [5].

Teeth agenesis can be divided into syndromic and non-syndromic. The discrimination of hypodontia on sporadic and familiar is basic for research purposes. Familiar agenesis can be divided on autosomal dominant hereditable, autosomal recessive and linked to X chromosome. The occurrence of this type of agenesis is observed also in other relatives. It is supposed that sporadic form of hypodontia is caused by multifactorial factors - environmental factors in the combination of more genes. Infections (e.g. rubella), some chemicals and drugs, radiotherapy, bone fractures etc. belong to environmental factors that can influence the pathogenesis of hypodontia.

Teeth agenesis arises as consequence of disbalance of epitelio-mesenchymal interactions, in which many signal ways are involved. More than 350 signal molecules in these processes are expected.

Odontogenesis research

Recent research of odontogenesis is based mainly on mouse models. On mouse model, the process of odontogenesis is analyzed - participation of signal molecules in given place and time. Histological slices of dentition in various differentiation stages are observed. Histological slices are possible to stain by special antibodies. Antibodies are stained by fluorescent colours and they are visible by using of fluorescent microscope. Specific antibodies are specific for the detection of separate signal molecules. It is possible to visualize this part of histological slice of developing dentition where signal way that use given signal molecule is in progress. When histological slices are prepared in different time of dentition differentiation, it is possible to observe sequential connecting of signal molecules in separate steps of differentiation.

vyřazen určitý gen. Lze pak zkoumat vliv tohoto genu na vývoj celého organizmu. Samozřejmě deplece některých genů je letální a zárodek se může vyvíjet jen do určitého vývojového stádia. Nicméně mnohé geny nejsou pro život nepostradatelné a u takových lze sledovat jejich vliv na vývoj orgánů a např. i dentice.

Výzkum odontogeneze na myším modelu odhalil mnohé geny, které se na vývoji dentice podílejí. Na základě získaných výzkumů bylo možné zahájit výzkum také u člověka. Zatímco pokusy s lidskými zárodky a jejich ovlivňování jsou z etického hlediska špatně obhajitelné a některé experimenty nelze vůbec provádět, lze studovat výskyt polymorfizmů a mutací genů, jejichž vliv na odontogenezi byl na myších experimentech prokázán. Mezi nejvíce studované geny ve vztahu k agenezi zubů patří geny pro PAX9, MSX1 a AXIN2. U těchto genů byl nalezen bezpočet mutací, které zřejmě ovlivňují vznik a vývoj zubní ageneze.

Výzkum ageneze zubů

Lidský genom obsahuje celkem 3,2 miliard párů bází, které jsou rozděleny ve 23 párech chromozómů. Každý chromozóm obsahuje část této genetické informace ve formě lineární molekuly DNA, která má dva konce a je navázána na bazické proteiny, tzv. histony. DNA se skládá mimo jiné ze čtyř typů nukleotidů, které jsou zjednodušeně nazývány adenin, thymin, guanin a cytosin. DNA má formu dvoušroubovice, ve které se báze komplementárně párují - adenin s thyminem a guanin s cytosinem. DNA obsahuje ve své sekvenci geny, tedy informace pro syntézu proteinů. Genetická informace je v DNA uložena ve formě primární sekvence DNA - tedy posloupnosti adeninu, tyminu, guaninu a cytosinu. V DNA lze číst jako v knize. Lze v ní rozeznávat jednotlivé geny, v genech lze určovat tzv. exony a introny. Exony jsou tou částí DNA, která kóduje primární strukturu proteinů, introny mají regulační funkce v procesu genové exprese. Proteiny se skládají z aminokyselin. Pořadí aminokyselin v sekvenci proteinu udává vlastnosti proteinu - především tvar molekuly a její funkce. U mutací a polymorfizmů DNA dochází k záměně nukleotidů v sekvenci DNA, např. k záměně adeninu za cytosin.

Pokud se taková záměna vyskytne v exonové části genu, je velká pravděpodobnost, že se změní také primární struktura proteinu a následně se může změnit také jeho funkce. Některé záměny nukleotidů v genech vedou ke vzniku stop kodónu. Potom buňka produkt daného genu vůbec nevyrobí (obdobný případ jako u knock-out myší). Některé mutace tedy vedou ke změně struktury proteinů, což zasahuje do jejich funkce v živé buňce. Protein může být díky mutaci jen zčásti funkční, může ho být méně, než je třeba v daný okamžik na daném místě a taková mutace může ovlivnit také průběh odontogeneze a vznik ageneze zubů.

Knock-out mice are also used. Mice missing specific gene is possible to prepare in the laboratory. It is possible to study the influence of this gene on ontogenesis of whole organism. Naturally, depletion of some genes is lethal and embryo can develop just till some developmental stage. However, many genes are not essential for the life and in these it is possible to observe their influence on dentition development.

The research of odontogenesis on mouse model revealed many genes that are involved in dentition development. On the basis of obtained researches it has been possible to initiate the research on humans. While experiments with human embryos and their influencing is from ethic point of view not justified and some experiments are not possible to carry out, it is possible to study the occurrence of polymorphisms and mutations of genes, whose influence on the odontogenesis was proved on mouse model. PAX9, MSX1 and AXIN2 genes are most studied genes in the relationship to teeth agenesis. In these genes, many mutations most probably influencing pathogenesis of teeth genesis were found.

The research of teeth agenesis

The human genome contains a total of 3,2 billions base pairs, that are dividend in 23 chromosome pairs. Each chromosome contains part of this genetic information in linear DNA molecule form that has two ends and is bounded to basic proteins named histons. DNA is composed beside others from four types of nucleotides that are named adenine, thymine, guanine and cytosine. DNA has form of double strand, in which bases are complementarily paired - adenine with thymine and guanine with cytosine. DNA obtains in her sequence genes - information for protein synthesis. Genetic information is deposited in DNA in the form of primary DNA sequence - sequence of adenine, thymine, guanine and cytosine. It is possible to read in DNA like in book. It is possible to recognize individual genes, in genes is possible to recognize exons and introns. Exons encodes primary structure of proteins, introns have regulatory function in gene expression. Proteins are composed from aminoacids. The order of amino acids in the sequence of a protein gives the properties of a protein - especially the shape of the molecule and its function. In mutations and polymorphisms of DNA, nucleotides changes in DNA sequence occur, for example adenine to cytosine change.

If such a change occurs in the exon part of the gene, there is a great likelihood that also the primary structure of the protein changes and its function can also change. Some of the substitutions of nucleotides in genes lead to the formation of stop codon. Than cell will not produce the product of this gene (a similar case

Obr. 1. Radiologický obraz vyvíjející se kompletní stálé dentice, dívka ve věku 9 roků. Počínající mineralizace třetích molárů v obou čelistech.
Fig. 1. Radiological picture of developing complete permanent dentition, a girl aged 9 years. Incipient mineralization of third molars in both jaws.

Výzkum ageneze zubů na souborech pacientů je tedy zaměřen na odhalování mutací v genech, jejichž vliv na odontogenezi byl v minulosti prokázán. Zkoumány bývají především exonové sekvence, které mohou obsahovat zcela zásadní mutace zasahující do genové exprese a tím i do vývoje dentice.

Jak takový výzkum probíhá?

Výběr pacientů pro mutační skríning provádí odborní zubní lékaři na základě klinického a radiologického vyšetření. Rozhodující pro zařazení jedinců do příslušných skupin studie je zhotovení aktuálního panoramatického rentgenového snímku obou čelistí, jeho analýza a klinické intraorální vyšetření. Vzhledem k vývoji dentice a zobrazování jednotlivých vývojových stadií zubů [6] je dolní věková hranice stanovena počínající mineralizací 3. molárů na 12. rok věku. Avšak oválné projasnění tvořené kostní kryptou můžeme u většiny pacientů pozorovat od 9. roku věku [7] (Obr. 1). Horní hranice není jednoznačně omezena, ovlivňují ji však extrakce stálých zubů, u dětí prováděné nejčastěji z ortodontických důvodů a to již od 10. roku věku. Do výzkumného projektu nejsou zařazováni jedinci, u kterých není možné stav chrupu radiologicky doložit.

Podle zjištěného zubního statusu jsou pacienti rozděleni do pěti skupin: 1. kompletní dentice (tj. včetně třetích molárů - kontrolní skupina), 2. ageneze třetích molárů (tj. chybí pouze jeden až všechny čtyři třetí moláry), 3. hypodoncie (chybí jeden až šest stálých zubů vyjma třetích molárů), 4. oligodoncie (chybí více než šest stálých zubů vyjma třetích molárů) a 5. rozštěpové vady (početní anomálie dentice u rozštěpů rtu a/nebo patra).

Pacientům je odebrán vzorek bukální sliznice pro izolaci DNA. Vzhledem ke skutečnosti, že se jedná o dětské pacienty, je výhodnější použít stěr z bukální sliznice, který pro pacienta nenese žádné riziko, je to neinvazivní a bezbolestná metoda jak získat vzorek pro DNA analýzy. Výhodou odběru bukální sliznice

as in the knock-out mice). Some mutations, therefore, lead to a change in the structure of proteins, which influences its function in the living cell. Protein may be due to a mutation only partly in the function, it may be less than needed at any given time on a given site. Such mutations can also affect the progress of odontogenesis and the formation of tooth agenesis. Research of tooth agenesis on patients is therefore focused on the detection of mutations in the genes, whose influence on odontogenesis has been in the past proved. Investigated are exon sequences that may contain crucial mutations affecting gene expression and in the development of dentition.

As such research take place?

Selection of patients for mutation screening is carried out by professional dentists based on clinical and radiological examination. Decisive for the inclusion of individuals in the study groups is making the current panoramic radiograph of jaws, its analysis and clinical intraoral examination. Due to the development of the dentition in and the display of individual developmental stages of teeth [6] is the lower age limit established by starting of mineralisation of 3th molars on 12 year olds. But the radiolucency of the oval formed by bone crypt can be observed in most patients from 9 year of age [7] (Fig.1). The upper age is not clearly limited; however, it is influenced by extraction of permanent teeth in children, carried out for most of the orthodontic reasons since the age of 10. Individuals, in that is not possible to prove the status of teeth radiologically, are not participating in the research project.

According to the dental status, patients are divided into 5 groups: 1. the complete dentition (i.e. including third molars - control group), 2. third molars agenesis (i.e., missing only one up to all four third molars), 3. hypodontia (missing one to six permanent teeth except the third molars), 4. oligodontia (missing more than six permanent teeth except the third molars), and 5. developmental defects (numerical anomalies of dentition in the clefts of the lip and/or palate). Samples of buccal swabs are sampled in patients for DNA isolation. Due to the fact that they are children's patients, it is preferable to use the sample of the buccal mucosa, in which the patient does not bear any risk, it is non-invasive and painless method to obtain a sample for DNA analysis. The advantage of the collection of the buccal mucosa on the special swabs is also the fact that it is not necessary to store tampons at a lower temperature, and after drying are stable for several weeks at room temperature. The problem may occur only in the laboratory, which has no experience with isolation DNA from buccal mucosa. It may contain a considera-

na speciální tampóny je také skutečnost, že tampony není nutné skladovat při nižší teplotě a po vysušení jsou stabilní po několik týdnů při pokojové teplotě. Problém může nastat pouze v laboratoři, která nemá zkušenosti s izolací DNA z bukální sliznice. Ta totiž může obsahovat značné množství kvasinek eventuelně PCR inhibitorů z potravy, z cigaret atd., které mohou následně působit nesnáze při DNA analýzách. V laboratoři je z tampónu izolována DNA buď manuálně pomocí komerčních izolačních metod, nebo lze použít i automaty pro izolaci DNA. U izolované DNA se stanovuje koncentrace a čistota.

Pro další analýzu jsou používány přístroje určené pro sekvenování DNA. Do nedávna převládaly v laboratořích tzv. kapilární sekvenátory, které umožňovaly analýzu souběžně až 96 vzorků, nicméně délka čtení se u těchto sekvenátorů pohybuje mezi 600-800 nukleotidy, takže pokud se gen skládá např. z pěti exonů o délce 1200 nukleotidů, pak je třeba provést i 10 sekvenačních reakcí. Přitom cena jedné sekvenční reakce se pohybuje okolo 200-250 Kč. Pokud je studováno více genů, pak se cena za analýzu DNA jednoho pacienta může vyšplhat i k desítkám tisíc Kč. Při našem výzkumu zjišťujeme, že v sekvencích mnohých pacientů s agenezí zubů se mutace v exonových sekvencích genů pro MSX1, PAX9 či AXIN2 nevyskytují. Je to dáno skutečností, že doposud nejsou známy a zkoumány všechny geny, které se na vzniku zubní ageneze podílejí.

Závěr

Nové světlo do výzkumu zubních agenezí by mohly vnést DNA sekvenátory nové generace. Ty totiž umožňují provádění sekvenčních analýz za přibližně desetinové náklady, což v praxi umožní výzkum většího počtu kandidátních genů a bude možné také studovat celé geny včetně intronů a regulačních oblastí.

Před několika lety na trh uvedené DNA čipové technologie umožnily tzv. celogenomové skríningy. Ty odhalily ve významném procentu v lidské populaci přítomnost tzv. copy number variations (CNV). Jedná se o úseky DNA dlouhé přibližně 1000 nukleotidů, které jsou v lidském genomu zaneseny v různých místech nebo naopak na některých místech chybějí, čímž dochází např. k duplikaci funkce některých genů nebo naopak k potlačení jejich funkce. Jako jeden z důsledků četných CNV v lidském genomu lze považovat také evoluci. Byl objeven vztah CNV ke kraniosynostóze [8]. Lze předpokládat, že také CNV a využití celogenomových analýz na DNA čipech by mohlo odhalit další příčinné mutace.

V posledních několika letech se často hovoří o nekódujících RNA (ncRNA) především ve vztahu ke kancerogenezi. Ukazuje se, že donedávna DNA označovaná jako nesmyslná je ve skutečnosti templářem pro tvorbu

ble amount of yeast or PCR inhibitors of food, cigarettes, etc., which may then cause trouble when performing DNA analyses. In the lab's is DNA isolated from swab either manually using commercial kits, or automats for isolation of DNA are used.

The concentration and purity of the isolated DNA is determined. For further analysis are used instruments designed for DNA sequencing. Until recently, prevailed in the laboratories capillary sequencers that allow analysis in parallel up to 96 samples, however the length of the reading is between 600-800 nucleotides, so if the gene consist for example of the five exons with a length of 1200 nucleotides, then you need to make 10 sequencing reactions. The price of one sequencing reaction is around 200-250 CZK. If more genes are studied, then the price for analysis of DNA of one patient can climb as well as tens of thousands of CZK. In our research, we find that in many patients with teeth agenesis a mutations in the exon parts of MSX1, PAX9 and AXIN2 genes do not occur. This is due to the fact that so far are not known and explored all of the genes that are involved in the formation of dental agenesis.

Conclusion

New light in research of dental agenesis are likely to bring a new generation of DNA sequencing instruments. These instruments allows sequencing analysis for approximately one-tenth the cost, which in practice will allow a larger number of research of candidate genes, and then allow also study the entire genes including introns and regulatory areas.

A few years ago on the market provided the DNA chip technologies allowed genome-wide screenings. Those revealed in a significant percentage of the human population the presence of copy number variations (CNV). This is a long DNA segments of approximately 1000 nucleotides, which are in the human genome entered in different locations, or on the contrary, in some places are missing, which occurs, for example to duplicate the function of certain genes or vice versa to inhibit their function. As a consequence of the numerous CNV in the human genome can be considered also the evolution. It was discovered the relationship of the CNV to craniosynostosis [8]. It can be assumed that also the CNV and the use of genome-wide analysis of the DNA chips could detect more causal mutations.

In the last few years, often talking about non-coding RNA (ncRNA), especially in relation to cancerogenesis. It turns out that until recently the DNA, known as the unsense DNA is in fact template for the creation of a huge amount of non-coding RNA, which plays a crucial role in cell regulation, particularly in the regulation of gene expression. Many of the polymorphisms,

obrovského množství nekódujících RNA, které hrají zcela zásadní roli v buněčných regulacích, především pak v regulaci genové exprese. Mnohé polymorfizmy, jejichž vliv na patogenezi některých onemocnění nebyl dostatečně chápán, ve světle ncRNA dostávají úplně nové funkce. Mnohé polymorfizmy vyskytující se mimo kódující úseky DNA ovlivňují totiž funkce ncRNA a zasahují tak do buněčných regulací. Také u hypodoncie lze předpokládat, že některé polymorfizmy mimo kódující úseky DNA mohou hrát roli v genových expresích. Výzkum v této oblasti se touto dobou teprve rozvíjí.

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whose impact on the pathogenesis of certain diseases has not been sufficiently understood, in the light of ncRNA have completely new features. Many of the polymorphisms occurring outside of the coding DNA segments affect the functions of ncRNA and extend into the cell regulations. Also in the hypodontia we can assume that some non-coding DNA segments polymorphisms may play a role in gene expression. Research in this area is developing at this time.

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Členský poplatek pro rok 2014 činí 2500,- Kč nebo 100,- EUR.

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Při nezaplacení příspěvků po dvou písemných urgencích bude ukončeno členství v ČOS.

Příloha IV – Sekvenace části genu pro *PAX9* a možná spojitost nalezených polymorfizmů s agenezí zubů

Sekvenace části genu pro PAX9 a možná spojitost nalezených polymorfizmů s agenezí zubů

Sequencing of the region of the PAX9 gene and possible connection between discovered polymorphisms and tooth agenesis

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Souhrn

Agenezí zubů rozumíme vrozený stav, kdy v dentici chybí jeden nebo více zubů. Tato porucha může mít celou řadu příčin - od působení lokálních změn v období zakládání zubních zárodků až po vliv především genetických faktorů. Polymorfizmus v genu, který kóduje protein zapojený do odontogeneze, může mít za následek poruchu jeho funkce, což může vývoj zubu negativně ovlivnit a v konečném důsledku úplně zastavit. Mezi nejvíce studované geny spojené se vznikem zubních agenezí patří geny pro PAX9, MSX1, AXIN2, WNT10a a EDA.

Cílem této studie bylo najít možný vztah mezi polymorfizmy v genu pro PAX9 a agenezí zubů na vzorku české populace. Analýza spočívala v DNA sekvenaci vybraných oblastí genu pro PAX9 a v následném porovnání výsledných sekvencí s referenční sekvencí z internetové databáze GenBank (NCBI).

Z výsledků námi provedené studie na české populaci se jeví jako nejvýznamnější inzerce 99-101insC (rs138135767, rs11373281) se současnou záměnou 272C>G (rs4904155; heterozygotní i homozygotní) v exonu 1 a varianty -54A>G (rs12882923), -41A>G (rs12883049) a 605C>T (Gly203Gly, rs61754301) v exonu 3, které budou dále studovány pro jejich možný vliv na ageneze zubů v české populaci (Ortodoncie 2014, 23, č. 1, s. 44-51).

Abstract

Under the term tooth agenesis we understand congenital absence of one or more teeth in the dentition. This disorder can have a variety of causes - from the influence of local causes during the formation of tooth germs primarily to the influence of genetic factors. Polymorphism in a gene, that encodes a protein involved in odontogenesis, may cause its malfunction, which may adversely affect the tooth development and eventually stop it completely. Among the most studied genes associated with the dental agenesis belong PAX9, MSX1, AXIN2, WNT10a and EDA genes.

The aim of this study was to examine the possible relationship between the PAX9 gene polymorphisms and tooth agenesis in Czech population. The analysis was based on DNA sequencing of selected regions of the PAX9 gene and consequent comparison of obtained sequences with the reference sequence from GenBank online database (NCBI).

The most important results of our study on Czech population seem to be insertion 99-101insC (rs138135767, rs11373281) with simultaneous substitution 272C>G (rs4904155; heterozygous and homozygous) in exon 1 and variants -54A>G (rs12882923), -41A>G (rs12883049) and 605C>T (Gly203Gly, rs61754301) in exon 3, that will be studied further for their possible effect on tooth agenesis in Czech population (Ortodoncie 2014, 23, No. 1, p. 44-51).

Klíčová slova: *odontogeneze, ageneze zubů, PAX9, sekvenace*
Key words: *odontogenesis, tooth agenesis, PAX9 gene, sequencing*

Úvod

Odontogeneze je velmi složitý a komplexní proces, na kterém se podílí přes 350 dosud identifikovaných proteinů, jež spolu navzájem recipročně interagují [1]. Transkripční a růstové faktory, signální molekuly aj. jsou těsně spjaty - regulují se navzájem a vytváří spletitou molekulární síť, která kontroluje a řídí celý proces odontogeneze [2]. I velmi malá změna v proteinu nebo regulačním procesu, který se účastní časoprostorové provázanosti jednotlivých proteinových interakcí, může mít za následek nezvratnou poruchu vývoje zubu [3].

Rozvoj molekulárně-biologických metod napomohl k nárůstu počtu studií molekulárních mechanizmů zásadních pro vývoj dentice. Tyto studie jsou prováděny především na myších. Výzkum výrazně přispěl k získání znalostí o časové lokalizaci exprese velkého množství genů zapojených do odontogeneze [1]. Vývoj probíhá i v oblasti genetiky a zároveň roste možnost jak identifikovat v lidském genomu geny nezbytné pro správný průběh odontogeneze, jejichž polymorfizmy by mohly mít za následek agenezi zubů [4, 5].

PAX9

Gen pro PAX9 (Paired box 9) se nachází na 14. chromozómu (14q-12-q13), obsahuje pět exonů a kóduje protein Pax9, který funguje jako transkripční faktor během embryogeneze, kdy se účastní diferenciace a udržování pluripotence buněčných populací. Během samotné odontogeneze je klíčový pro postupnou a vzájemnou interakci mezi ústním epitelem a mezenchymem, který je tvořen buňkami, jež vycestovaly z neurální lišty [6, 7, 8].

Protein Pax9 je složen z 341 aminokyselin, obsahuje DNA-vazebnou doménu na N-konci, transkripčně regulační doménu na C-konci, která je bohatá na alanin (13,7 %), serin (13,2 %), prolin (12,7 %) a glycin

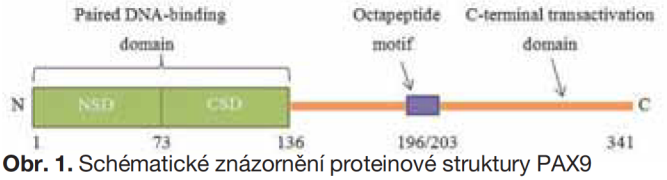
Introduction

Odontogenesis is very complicated and complex process involving over 350 so far identified proteins that interact each other [1]. Transcription factors and growth factors, signalling molecules and others are closely linked - they regulate each other and create a complex system of regulatory linkages which monitors and controls the entire process of odontogenesis [2]. Even a very small change in the protein or regulatory process that is involved in spatio-temporally cohesion between the individual protein interactions, can result in irreversible disorder of tooth development [3].

The development of molecular-biological techniques has led to increase of number of studies of molecular mechanisms involved in the development of dentition. Studies are carried out mainly on mouse models. This research significantly contributed to the knowledge of temporal localization of the expression of large number of genes involved in odontogenesis [1]. Development is in progress in the field of genetics too and simultaneously increases the possibility to identify genes in the human genome that are necessary for the correct progress of odontogenesis whose polymorphisms could result in tooth agenesis [4, 5].

PAX9

PAX9 (Paired box 9) gene is located on the 14th chromosome (14q-12-q13), it contains 5 exons and encodes Pax9 protein. Pax9 protein acts as a transcription factor important for embryogenesis, during it Pax9 participates in differentiation and maintenance of pluripotence of cell populations. During odontogenesis Pax9 is essential for progressive and reciprocal interactions between oral epithelium and mesenchyme, which is made up of cells derived from the neural crest [6, 7, 8].



Obr. 1. Schématické znázornění proteinové struktury PAX9
Fig. 1. Schematic illustration of PAX9 protein structure
NSD = N-terminal subdomain; CSD = C-terminal subdomain; numbers show amino acid sequence

(8,3 %), a oktapeptidový motiv. DNA-vazebná doména zprostředkovává rozpoznání a vazbu na sekvenčně specifické motivy DNA, což umožňuje aktivovat nebo potlačovat expresi cílových genů. Tato doména je složena ze dvou subdomén, N-koncové a C-koncové, spojených krátkým segmentem. Každá ze subdomén obsahuje tři α-šroubovice (v případě N-subdomény jsou přítomny navíc ještě dva β-řetězce), které umožňují vazbu na DNA [9] (Obr. 1).

Exprese Pax9 je markerem časné fáze odontogeneze a předchází expresi dalších genů [10]. Ve fázi zubního pupenu i během přechodu do následujícího stadia zubní čepičky interaguje s Msx1 (Muscle segment homeobox 1) a Bmp4 (Bone morphogenetic protein 4) [11]. Během stádia čepičky je Pax9 potřebný pro kondenzaci zubního mezenchymu, okolo něhož obrůstá ústní epitel. Pokud je funkce Pax9 narušena, dochází v mezenchymu ke snížení exprese klíčových odontogenních molekul a vývoj zubu se zastaví ve stádiu pupenu [12]. Vysoká úroveň exprese Pax9 tedy přetrvává v zubním mezenchymu během stádia pupenu a pohárku, poté se během stádia zubního zvonku snižuje [10].

Vzhledem k tomu, že Pax9 působí jako transkripční faktor, mohou polymorfizmy v tomto genu ovlivnit mnohé funkce a procesy, jako je schopnost vazby na DNA, transkripční aktivita nebo synergistická interakce s koaktivátory, jakým je například výše zmíněný Msx1 [9]. Za hlavní patologickou událost je pokládáno právě narušení DNA-vazebné funkce [13]. Dosud bylo publikováno přes 30 polymorfizmů v genu pro PAX9, které byly asociovány s agenezemi zubů.

Materiál a metodika

Cílem této studie bylo nalezení polymorfizmů a mutací lokalizovaných v kritických oblastech genu pro PAX9 u osob s agenezí zubů z české populace. Pro stanovení přítomnosti polymorfizmů v genu pro PAX9 byly vybrány první tři exony a přilehlé intronové oblasti, z důvodu největší četnosti polymorfizmů a mutací souvisejících s agenezí zubů doposud publikovaných v literatuře. Exon 1 je celý netranslatován (5'-UTR oblast), ale předpokládá se, že může během translace uplatňovat regulační funkci. Exon 2 obsahuje zbývající 5'-UTR oblast, pouze na jeho konci se nacházejí čtyři báze, které jsou translatovány (tj. iniciační kodon a první

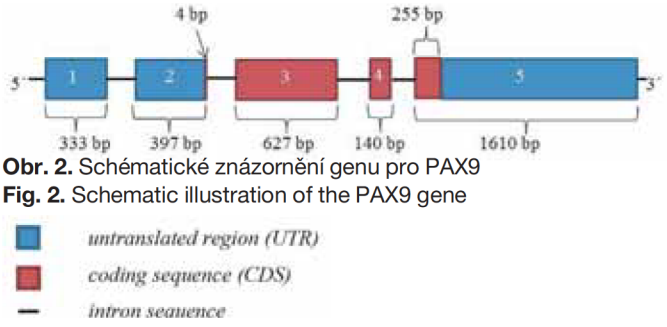
Pax9 protein is composed of 341 amino acids, it contains the DNA-binding domain at the N-terminus, transcriptional regulatory domain at the C-terminus, that is rich in alanine (13,7 %), serine (13,2 %), proline (12,7 %) and glycine (8,3 %), and the octapeptide motive. DNA-binding domain mediates recognition and binding on the sequence-specific DNA motives, which allows to activate or inhibit the expression of target genes. This domain is composed of two subdomains, the N-terminal subdomain and the C-terminal subdomain, connected with a short segment. Each of the subdomains contains three α-helices (two β-chains are present in the case of the N-subdomain additionally) that allow DNA binding [9] (Fig. 1).

Expression of PAX9 gene is a marker of early stadium of odontogenesis and it occures before the expression of other genes [10]. In the bud stage and during the transition to the next cap stage too, Pax9 interacts with Msx1 (Muscle segment homeobox 1) and Bmp4 (Bone morphogenetic protein 4) [11] During the cap stage Pax9 is required for dental mesenchyme condensation, around which the oral epithelium overgrows. If the Pax9 function is impaired, the expression of key odontogenic molecules decreases in the mesenchyme and tooth development stops at the bud stage [12]. High level of expression of Pax9 thus persists in the dental mesenchyme during the bud stage and the cup stage, then it decreases during the bell stage [10].

Given that Pax9 acts as a transcription factor, polymorphisms in this gene may affect many functions and processes, such as the ability of DNA binding, transcriptional activity or synergistic interactions with co-activators, such as Msx1 mentioned above [9]. Disruption of DNA-binding function is thus considered as the main pathological event [13]. To date over 30 polymorphisms in the gene for PAX9 have been presented that have been associated with tooth agenesis.

Material and methods

The aim of this study was to find which polymorfisms and mutations are located in critical regions of the PAX9 gene in individuals with tooth agenesis in the Czech population. We selected first three exons and adjacent intronic region to determine the presence of polymorphisms in the PAX9 gene because of the largest published frequency of mutations associated with tooth agenesis in these critical areas. The exon 1 of PAX9 gene is not translated (5'-UTR region), but it is supossed, that exon 1 may influence its regulatory function during translation. Exon 2 is the next part of the 5'-UTR region and at the end of this region there are four bases which are translated (ie, an initiation codon and the first base of the next encoding triplet).



Obr. 2. Schématické znázornění genu pro PAX9
Fig. 2. Schematic illustration of the PAX9 gene

báze z následujícího kódujícího tripletu). Exon 3 je celý překládán, tato oblast kóduje DNA-vazebnou doménu. Zbývající 4. a 5. exon prozatím nebyly v naší studii analyzovány (Obr. 2).

Spolu s kódujícími oblastmi byly analyzovány přilehlé intronové oblasti. Polymorfizmům v intronech nebývá přikládán velký význam z toho důvodu, že jsou během posttranskripčních úprav vystřiženy. V poslední době bylo ovšem zjištěno, že introny (nc-RNA) mohou mít významný vliv na sestřih a přítomné polymorfizmy mohou tento proces pozměnit, proto je vhodné těmto oblastem věnovat pozornost [4].

Zkoumaný soubor tvořilo 97 pacientů s agenezí zubů a 6 osob s kompletní denticí, jež sloužily jako kontrolní vzorky. Diagnóza ageneze zubů byla provedena pomocí rentgenových snímků. Vzorky byly zasílány ze zubních klinik v Brně (doc. MUDr. Černochová P., Ph.D.), Ostravě (MUDr. Štembírek J., Ph.D.) a Olomouci (prim. MUDr. Krejčí P., Ph.D.).

DNA pacientů byla izolována z krve nebo bukálních stěrů pomocí izolačního kitu UltraClean BloodSpin DNA (Mo-Bio). Exony 1 a 3 byly amplifikovány specifickými primery k těmto oblastem pomocí KAPA2G Fast HotStart ReadyMix (Kappa Biosystems). Produkty PCR pro exon 2 byly připraveny pomocí modifikované polymerázy Kapa 2G Robust Hot Start (Kapa Biosystems) s přídavkem DMSO vzhledem k vysokému podílu GC oblastí v DNA sekvenci. Pro zaručení specifity byly navrhnuty sekvenační primery. Amplikony byly sekvenovány pomocí genetického analyzátoru ABI 3130 Prism (Life Technologies). Výsledné sekvence byly porovnány se standardní sekvencí genu pro PAX9 [14] v programu BioEdit v.7.0.8.0 [15] a polymorfizmy byly popsány z hlediska jejich možného vlivu na proteinovou strukturu.

Výsledky

V celkovém souboru 97 pacientů byly nejčastěji chybějícím zubem třetí moláry, následované druhými dolními premoláry a postranními horními řezáky. Soubor se skládal z 59 žen a 38 mužů.

V exonu 1 se u kontrolních osob s plnou denticí i u pacientů vyskytovala heterozygotní/homozygotní inzerce cytosinu v nukleotidové pozici 99-101insC (rs138135767, rs11373281). Kvůli dvěma cytosinům

Entire exon 3 is translated and encodes a DNA-binding domain. Remaining exon 4 and exon 5 have not yet been analyzed in this study (Fig. 2).

Intronic regions, that are adjacent the coding regions, were analyzed too. Intronic polymorphisms are usually not of major importance because they are cut out during the post-transcriptional modifications. Lately, however, was found that introns may have a significant influence during the regulation of gene expression through ncRNA (non-coding RNA). Intronic polymorphisms can control gene expression through ncRNA influence and therefore it is advisable to pay attention to these areas [4].

The sample consisted of 97 patients with tooth agenesis and 6 subjects with complete dentition as the control group. Diagnosis of dental agenesis was performed using X-ray images. Samples were sent from dental clinics in Brno (doc. MUDr. Černochová P., Ph.D.), Ostrava (MUDr. Štembírek J., Ph.D.) and Olomouc (prim. MUDr. Krejčí P., Ph.D.).

DNA of patients was isolated from blood or buccal swabs using isolating kit UltraClean BloodSpin DNA (MoBio). Exons 1 and 3 were amplified with primers specific to these areas by KAPA2G Fast HotStart Readymix (Kapa Biosystems). The PCR products for exon 2 were prepared using a modified polymerase KAPA2G Robust HotStart (Kapa Biosystems) containing DMSO due to the high content of GC regions in the DNA sequence. Special sequencing primers were designed to ensure specificity. The amplicons were sequenced using a genetic analyzer ABI PRISM 3130 (Life Technologies). Obtained sequences were compared with the standard sequence of PAX9 gene [14] in the BioEdit v.7.0.8.0 software [15] and polymorphisms have been described from the poin of view of their possible effects on protein structure.

Results

The most frequently missing teeth were third molars, followed by second lower premolars and second upper incisors in the group of 97 patients. The group comprised of 59 women and 38 men.

We found the heterozygous/homozygous insertion of cytosine at the nucleotide position 99-101insC (rs138135767, rs11373281) in exon 1 in control subjects with complete dentition and in group of patients. We did not accurately determined the position of insertion of cytosine due to the occurrence of two adjacent cytosines.

The heterozygous substitution 272C>G (rs4904155) occurred simultaneously with the insertion 99-101insC in 4 samples and the combination of the homozygous insertion 99-101insC occurred simultaneously with the homozygous substitution at po-

vedle sebe jsme přesně nestanovili polohu inzerce cytosinu.

Heterozygotní záměna 272C>G (rs4904155) se vyskytovala současně s inzercí 99-101insC u čtyř vzorků a kombinace homozygotní inzerce 99-101insC se vyskytovala společně s homozygotní substitucí v pozici 272C>G u devatenácti vzorků. Nejčastěji chybějícími zuby u těchto 19 jedinců byly třetí moláry v prvním kvadrantu, následované agenezí třetích molárů ve zbývajících kvadrantech. Dalšími chybějícími zuby byly dolní druhé moláry a dolní druhé premoláry ve třetím a čtvrtém kvadrantu.

U tří osob se společně s inzercí 99-101insC vyskytl polymorfizmus -40A>C. Objevovala se i heterozygotní inzerce 99-101insC se současnou heterozygotní záměnou 272C>G a -40A>C, ovšem pouze v několika ojedinělých případech.

V exonu 2 nebyl nalezen žádný polymorfizmus, pouze standardní sekvence u pacientů i kontrolních vzorků.

Ve třetím exonu byly nejčastěji nalezeny polymorfizmy -54A>G (rs12882923) a -41A>G (rs12883049), a to jak samostatně, tak také v kombinacích, a to u pacientů i kontrolních osob. Oba polymorfizmy se nacházejí v nekódující oblasti předcházející exonu 3 na pozici vzdálené 54, resp. 41 bp od prvního nukleotidu exonu 3. U tří pacientů, z toho dvou příbuzných, byl nalezen polymorfizmus 605C>T (rs61754301), který se nachází na nukleotidové pozici 605, kódovanou 203. aminokyselinou však zůstává glycin. Ostatní sekvence byly standardní.

Závěr a diskuse

Nejčetněji nalézanými polymorfizmy na souboru pacientů (19 osob z 97) byly kombinace homozygotní inzerce cytosinu 99-101insC s homozygotní záměnou 272C>G. Oba polymorfizmy se nachází v 5'-netranslatující se oblasti (5'-UTR), která může hrát roli v regulaci genové exprese, např. ovlivňovat transkripci, posttranskripční úpravy RNA nebo translaci [16, 17]. U většiny pacientů se vyskytovala ageneze třetích molárů, dalšími často chybějícími zuby byly dolní druhé moláry a za zmínku stojí i ageneze dolních druhých premolárů. Byla nalezena i heterozygotní varianta těchto dvou současně se nacházejících polymorfizmů.

Protože se tyto polymorfizmy vyskytovaly u pacientů i osob s kompletní denticí, nemůžeme určit, zda mají vliv na vznik agenezí zubů nebo tvoří pouze interindividuální rozdíl bez závažných následků. Zajímavostí je, že se tyto dvě odchylky v sekvenci genu pro PAX9 vyskytují vždy současně a to ve všech našich zjištěných případech. U vzorků pacientů byly kromě výše zmíněných polymorfizmů nalezeny i další intronové záměny a kombinace, které se ale vyskytují pouze v několika

sition 272C>G in 19 samples. Third molars in the first quadrant, followed by agenesis of third molars in the remaining quadrants, were the most commonly missing teeth in this group of 19 subjects. Mandibular second molars and mandibular second premolars in the third and fourth quadrant were other frequently missing teeth.

The insertion 99-101insC occurred with the polymorphism -40A›C together in 3 people. The heterozygous insertion 99-101insC occurred with the heterozygous substitution 272C>G and -40A>C, but only in a few sporadic cases.

We found no polymorphism in exon 2 in patients and controls, just the standard sequences.

The polymorphism -54A>G (rs12882923) and -41A>G (rs12883049), both individually and in combinations, both in patients and in control subjects, were the most commonly polymorphisms in exon 3. Both polymorphisms are located in the noncoding region before exon 3 at position distant 54, respectively 41 bp from the first nucleotide of exon 3. In 3 patients, including 2 related, we discovered the polymorphism 605C>T (rs61754301), located at the coding nucleotide position 605, the coded 203th amino acid remains glycine. Other sequences were standard.

Conclusion and discussion

The homozygous combination of insertion cytosine 99-101insC with the homozygous substitution 272C>G were the most frequently found polymorphisms in group of patients (19 of 97 cases). Both polymorphisms are located in the 5'-untranslated region (5'-UTR), which may play a role in regulation of gene expression, such as to influence the transcription, post-transcriptional modifications of RNA or translation [16, 17]. Most patients suffered from agenesis of third molars, mandibular second molars and noteworthy mandibular second premolars agenesis were other frequently missing teeth. We found the heterozygous variant of these two simultaneously located polymorphisms. We cannot consider if these polymorphisms they comprise just interindividual variance without significant consequence, because these polymorphisms occurred in patients and in persons with complete dentition. It is interesting, that these two polymorphisms always occur in the sequence of PAX9 gene simultaneously in all cases. In patient samples, except the above-mentioned polymorphisms, we found other intronic substitutions and combinations, that occur but only in a few sporadic cases, and their possible relationship with tooth agenesis we could check in further analyzes on a larger group of patients.

During the study of literary sources we were able to determine that no study concerned with the exon 1 of

lika ojedinělých případech a jejich možný vztah s agenezí zubů by se mohl prokázat při dalších analýzách na větším souboru pacientů.

Při studiu literárních zdrojů se nám podařilo zjistit, že exonem 1 genu pro PAX9 se žádná studie dosud nezabývala, a proto mohou i tyto dílčí výsledky být přínosem pro další výzkum. Díky analýze výsledků rozličných zahraničních studií jsme zjistili, že v publikacích dochází k nesprávnému číslování exonů, kdy je exonem 2 myšlen ve skutečnosti exon 3.

Nejvýraznějším výsledkem bylo nalezení polymorfizmu v kódující oblasti 605C>T (Gly203Gly) u 2 pacientů s agenezí třetích molárů ve dvou kvadrantech a u 1 pacienta s agenezí třetích molárů ve všech kvadrantech a druhých premolárů ve dvou kvadrantech.

Dalšími identifikovanými polymorfizmy v exonu 3 byly intronové záměny -41A>G a -54A>G, které se vyskytovaly i u kontrolních vzorků. Polská studie Pawlowské, et al. [18] asociovala tyto dva polymorfizmy s možným vlivem na ageneze zubů. V naší studii jsme tyto polymorfizmy nalezly také u osob s kompletní denticí, takže lze vyloučit přímý efekt těchto polymorfizmů na dentální agenezi.

Při srovnání našich výsledků s výsledky ostatních zahraničních studií provedených na genu pro PAX9 jsme nedošli k žádné výrazné shodě. Právě exonu 3 bývá věnována zvýšená pozornost, protože je v této oblasti kódována DNA-vazebná doména, která umožňuje proteinu fungovat jako transkripční faktor a narušení její funkce je obecně pokládáno za hlavní patologickou událost způsobující zubní ageneze.

Ačkoliv bylo v oblasti exonu 3 popsáno větší množství polymorfizmů, na vzorku české populace jsme tato data prozatím nepotvrdili a naopak, námi odhalené varianty nebyly zatím v žádné studii popsány. Vysvětlením tohoto rozporu by mohla být skutečnost, že se populace osob s agenezí zubů v České republice geneticky liší od doposud studovaných populací. Tento nález poukazuje na důležitost provádění výzkumu genetické podmíněnosti vzniku zubních agenezí v různých populacích.

I u pacientů, u kterých byla potvrzena zubní ageneze, se vyskytovaly standardní nukleotidové sekvence. Vývoj zubu je řízen expresí genů velkého počtu proteinů, které nebyly dosud analyzovány. Předpokládáme tedy vliv dalších genůna agenezi zubů ve skupině našich pacientů.

V dalším výzkumu bude naše pozornost zaměřena na sekvenační analýzy genů pro MSX1 a AXIN2. Bude zvětšen soubor vzorků pacientů i kontrolních vzorků, abychom mohli statisticky vyhodnocovat možné asociace.

Rodinné studie se jeví jako jedna z efektivních metod pro ověřování vztahu nově identifikovaných mutací s agenezí zubů.

PAX9 gene, so our partial results could contribute for further research. Thanks to analyzing of results of various publications we found incorrect numbering of exons in these publications, when there exon 2 means the third exon in the fact.

As the most interesting result we consider discovering of the polymorphism 605C>T (Gly203Gly) in the coding region in 2 patients with agenesis of the third molars in two quadrants and 1 patient with agenesis of the third molars in all quadrants and second premolars in two quadrants.

The intron substitutions -41A>G and -54A>G were other identified polymorphisms in exon 3, but these polymorphisms occurred even in the control samples. Polish study published by Pawlowská et al. [18] associate these two polymorphisms with possible influence on tooth agenesis. In our study we also found these polymorphisms in persons with complete dentition, so it is possible to exclude direct effect of these polymorphisms on the dental agenesis.

When comparing our results with other international studies carried out on PAX9 gene, we did not reach any significant agreement. Special attention is given to exon 3 because DNA-binding domain is coded in this area and this domain allows to act the protein as a transcription factor and disruption of its function is generally regarded as the main pathological cause of tooth agenesis.

Although more polymorphisms were described in exon 3, these data are not being confirmed on the sample of Czech population and on the other hand, we detected polymorphisms that were not described in any of the studies. The explanation of this difference could be the fact that the population of people with tooth agenesis in Czech Republic differ from previously studied populations genetically. This finding highlights the importance to conduct research of genetic conditions of dental agenesis in different populations.

Standard nucleotide sequences also occured in patients with confirmed dental agenesis. Tooth development is controlled by expression of a large number of genes that have not been analyzed yet. Therefore we suppose the influence of other genes on dental agenesis in our group of patients.

In further research we will focus our attention on sequence analysis of MSX1 and AXIN2 genes. We will enlarge the group of patients and control subjects in order to be able statistically evaluate possible associations.

Familiar studies are one of effective methods for checking of association of newly identified mutations in relationship with teeth agenesis.

The most frequent absence of molars followed by premolars follows from our study from the perspective

Z pohledu srovnání morfologické třídy vyplývá z naší studie nejčastější absence molárů, následovaných premoláry. V tom se naše výsledky shodují s výsledky jiných studií.

Velmi často se v našem souboru pacientů objevuje bilaterální absence zubů, v čemž se rovněž shodujeme s českými i zahraničními studiemi, např. Symons et al. [19], Závadová [20], Heringová et Černochová [21].

Prevalence ageneze se liší podle pohlaví. Naše výsledky uvádějí 59 žen a 38 mužů, u žen je tedy na základě této studie výskyt 1,55x vyšší. Pro srovnání se známými daty, prevalence je uváděna taktéž vyšší u žen (v poměru ženy:muži 3:2), u žen je udáván výskyt 1,37x vyšší než u mužů [12].

Závěrem, z výsledků námi provedené studie na české populaci se tedy jeví jako nejvýznamnější inzerce 99-101insC se současnou záměnou 272C›G (rs4904155; heterozygotní i homozygotní) v exonu 1 a varianty -54A›G (rs12882923), -41A›G (rs12883049) a 605C›T (Gly203Gly, rs61754301) v exonu 3, které budou dále studovány pro jejich možný vliv na ageneze zubů v české populaci.

Autoři nemají komerční, vlastnické nebo finanční zájmy na produktech nebo společnostech popsaných v tomto článku.

of comparison of morphological class. In these results we are in agreement with the results of other studies.

In our group of patients bilateral absence of teeth appears very often, that also corresponds with the Czech and foreign studies, such as Symons et al. [19], Závadová [20], Heringová et Černochová [21].

The prevalence of agenesis differs by gender. Our results show 59 women and 38 men, in group of women the incidence is 1,55 times higher based therefore on this study. When we compare our results with the known data, prevalence is reported higher in women too (ratio of female:male 3:2), incidence in women is reported 1,37 times higher than for men [12].

In conclusion, from the results of our study on Czech population, the most important result is the insertion 99-101insC with the simultaneously substitution 272C›G (rs4904155, heterozygous and homozygous) in exon 1 and the polymorphisms -54A›G (rs12882923), -41A›G (rs12883049) and 605C›T (Gly203Gly, rs61754301) in exon 3 which will be further studied for their possible effect on tooth agenesis in Czech population.

The authors have no comercial, proprietary, or financial interests in the products or companies described in this article.

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Termín: **16.–17. 5. 2014**
Místo konání: **Angelo hotel Prague, Radlická 1g, Praha 5**
Téma: **Biomechanika a estetika založená na strategii ortodontické léčby**
Přednášející: Prof. Dr. Ravi Nanda

Těší se na Vás kolektiv Altis Group s.r.o.

Altis Group spol. s r. o., Žerotínova 901/12, 690 02 Břeclav
Tel./fax: 519 325 414, e-mail: ortho@altisgroup.cz, Petra Karafová - 731 476 456, Marie Písaříková – 606 746 716
Zelená linka: 800 100 535 (VOLEJTE ZDARMA!)

LÉČBA MALOKLUZÍ III. TŘÍDY

Termín: 30.5.2014, 9:00 h (pátek)
Místo konání: Praha: budova Dentamed (Pod Lipami 41, Praha 3)

Na četná přání účastníků loňského školení jsme pozvali do Prahy opět prof. Nazan Kucukkeles. Školení na téma „Vertikální problematika od A do Z" mělo u Vás velký ohlas. Rádi bychom Vám nabídli nové téma „Léčba malokluzí III. třídy".

- malokluze III. třídy
- léčba hraničních případů III. tříd fixními mechanikami
- léčba retrognácie maxily pomocí reverzního extraorálního tahu
- RME s podporou reverzního extraorálního tahu
- distrakční osteogeneze
- distrakce maxily: intraorální a extraorální
- distrakce maxily pomocí obličejové masky a elastiků
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- chirurgická korekce malokluzí III. třídy

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Příloha V – Novel *PAX9* gene polymorphisms and mutations and susceptibility to tooth agenesis in the Czech population

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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´-agctcccttctcttaaaatcaga-3´	5´-tcgagtcattcacattcaga-3´
	3	5´-gattggacagtgacggtttg-3´	5´-ggaaagacagtgtccctgag-3´	5´-ggacagccccagtagttagt-3´

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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)

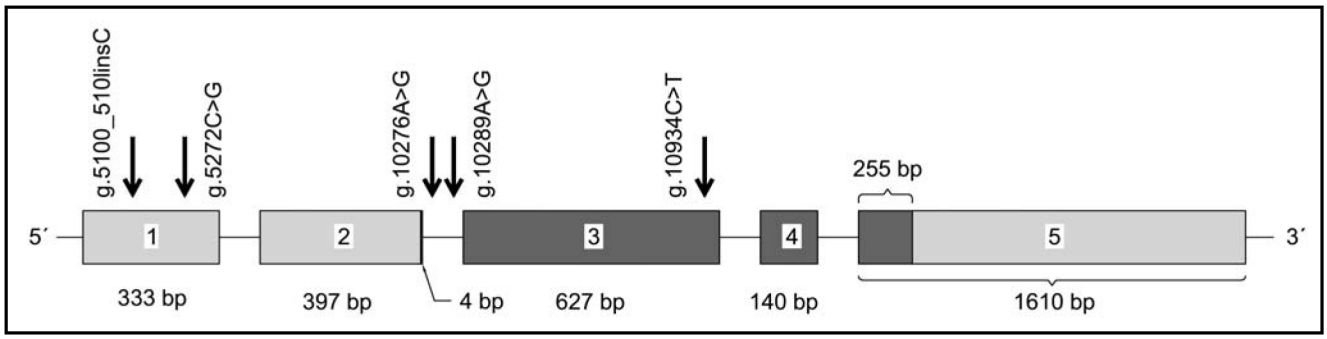

Fig. 1. Schematic illustration of the PAX9 gene and found mutations grey colour – untranslated region (UTR); dark grey colour – coding sequence (CDS).

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-

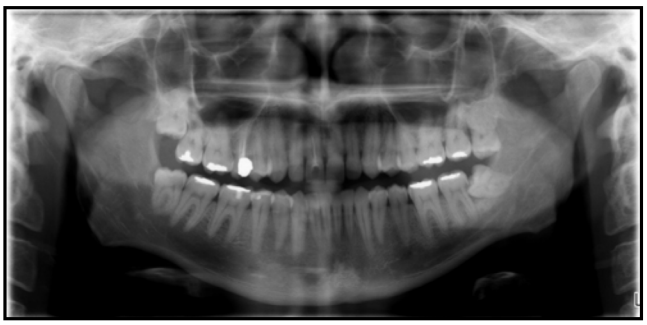

Fig. 2. Panoramic radiograph of unaffected father proband (full dentition, dislocation 38).

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).

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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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Příloha VI – A screen of a large Czech cohort of oligodontia patients implicates a novel mutation in the *PAX9* gene

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A screen of a large Czech cohort of oligodontia patients implicates a novel mutation in the *PAX9* gene

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Tooth agenesis is one of the most common developmental anomalies in humans. To date, many mutations involving paired box 9 (*PAX9*), msh homeobox 1 (*MSX1*), and axin 2 (*AXIN2*) genes have been identified. The aim of the present study was to perform screening for mutations and/or polymorphisms using the capillary sequencing method in the critical regions of *PAX9* and *MSX1* genes in a group of 270 individuals with tooth agenesis and in 30 healthy subjects of Czech origin. This screening revealed a previously unknown heterozygous g.9527G>T mutation in the *PAX9* gene in monozygotic twins with oligodontia and three additional affected family members. The same variant was not found in healthy relatives. This mutation is located in intron 2, in the region recognized as the splice site between exon 2 and intron 2. We hypothesize that the error in pre-mRNA splicing may lead to lower expression of PAX9 protein and could have contributed to the development of tooth agenesis in the affected subjects.

Tooth agenesis is one of the most common developmental anomalies in humans, affecting approximately 20% of the world's population (1). When agenesis of fewer than six teeth is present, it is described as hypodontia, and when more than six teeth (excluding third molars) are missing, the condition is referred to as oligodontia. Oligodontia is rare in the general population, with an estimated prevalence of 0.08–0.16% (2, 3).

Genetic defects responsible for human tooth agenesis are only recently beginning to be uncovered. So far, most of the mutations related to tooth agenesis have been found in the msh homeobox 1 (*MSX1*) and paired box 9 (*PAX9*) genes (4–7). The *MSX1* gene encodes a protein that belongs to a family of transcription factors expressed in overlapping patterns at multiple sites during vertebrate development. The MSX1 protein co-determines the position and shape of teeth. The *PAX9* gene encodes a member of the paired box family of transcription factors and it is widely expressed in the neural crest-derived mesenchyme involved in craniofacial and dental development. The PAX9 protein plays an important role as a regulator of cellular pluripotency and differentiation during embryonic patterning and organogenesis and in postnatal life. The most important events during the regulation of tooth development are inductive interactions between the epithelial and mesenchymal tissues (8). *MSX1* and *PAX9* genes display sequential and reciprocal signaling interactions rather than a one-way process (4). Mutations in these two genes have been suggested to cause selective tooth agenesis: mutations in the *MSX1* gene have been specifically associated with second-premolar and third-molar agenesis, whereas *PAX9* gene mutations have been linked to the absence of permanent molars, in some instances also accompanied by the absence of upper lateral incisors and premolars (9, 10).

The aim of the present study was to perform screening for mutations and/or polymorphisms in the critical regions of the *PAX9* and *MSX1* genes in 270 patients with oligodontia, 97 of whom had been screened in a pilot study conducted by Hloušková *et al.* (11). The inclusion of 30 healthy subjects enabled us to distinguish between polymorphisms and mutations commonly present in the Czech population and those specific for tooth agenesis. Subsequently, in twins with a newly identified mutation, a family study was performed.

Material and methods

A total of 300 study subjects (270 individuals with tooth agenesis and 30 unrelated healthy control subjects) were enrolled in the Clinic of Stomatology of St. Anne's Faculty Hospital in Brno and in the Institute of Dentistry and Oral Sciences in Olomouc. The age of patients and controls ranged from 9 to 23 yr. Twins from a four-generation family

with multiple affected family members were among the individuals with tooth agenesis included in the study.

Oral panoramic radiographs (Planmeca ProMax 3D; Planmeca, Helsinki, Finland) were taken in all study subjects and controls. Dental phenotypes were assessed by oral examination and inspection of dental radiographs using the PLANMECA ROMEXIS software (version 2.9.2; Planmeca). The study protocol and informed consents were reviewed and approved by the ethical committees of both Faculty Hospitals involved in the study. Written informed consent was obtained from all study participants.

DNA analysis

Buccal swab samples for genomic DNA isolation were collected from all study subjects, controls, and family members. DNA was isolated using a fully automated instrument, chemagic Prepito (PerkinElmer, Waltham, MA, USA) and a paramagnetic bead-based protocol. DNA quality and concentration were checked by spectrophotometry. Genomic DNA was used for capillary DNA sequencing using the automated ABI 3130 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA).

First of all, we selected three exons and adjacent intronic regions to determine the presence of polymorphisms and mutations in the *PAX9* gene. Exon 1 is not translated and has not been previously investigated in relation to oligodontia/hypodontia. Exon 2 contains the start codon and the first nucleotide that is part of a triplet encoding the first amino acid. In this exon, just two mutations have been described (12, 13). In exon 3, a total of 21 mutations have been found, whereas in exons 4 and 5 only three mutations have been reported (14–16); for this reason, we did not include exons 4 and 5 in our screening.

Entire exon sequences, including intron–exon boundaries for exons 1–3 of the *PAX9* gene and exons 1 and 2 of the *MSX1* gene, were amplified by PCR in a 20-μl reaction volume. Specific PCR primers were used for genomic DNA amplification (Table 1).

The PCR amplifications of exons 1 and 3 of the *PAX9* gene were carried out using the KAPA2G Fast HotStart ReadyMix (Kappa Biosystems, Wilmington, MA, USA). For exon 2 of the *PAX9* gene, the KAPA2G Robust HotStart kit (Kappa Biosystems) plus 5% dimethyl sulphoxide (DMSO) were used. The conditions for PCR were: an activation/denaturation step at 95°C for 2.5 min; 45 cycles at 95°C for 30 s and 58°C (for exons 1 and 3) or 54°C (for exon 2) for 30 s; and 72°C for 60 s, and a final extension for 7 min.

All PCR analyses of exons 1 and 2 of the *MSX1* gene were performed using the KAPA2G Robust HotStart kit (Kappa Biosystems), 5% DMSO, and 1% 7-Deaza-dGTP (Roche Diagnostics, Basel, Switzerland). Robust polymerase, DMSO, and 7-Deaza-dGTP were used for PCR mixtures because of the very high content of G + C bases in the nucleotide structure of the *MSX1* gene and in exon 2 of the *PAX9* gene. The PCR conditions were: a 10-min activation/denaturation step at 95°C; 40 cycles at 95°C for 60 s, 58°C (with 20% ramp) for 30 s, and 72°C for 60 s; and a final extension for 7 min. The Veriti® thermal cycler (Applied Biosystems, Wilmington, MA, USA) was used for all PCR reactions.

Amplicons were purified by ExoI-FastAP (Fermentas, Waltham, MA, USA). The mixtures were incubated at 37°C for 15 min and subsequently at 85°C for 15 min to inactivate the enzymes. Sequencing was performed using BigDye® Terminator v.3.1 (Life Technologies). Sequencing reactions/products were purified using ethylenediaminetetraacetic acid (EDTA)/ethanol precipitation, resuspended in 10 μl of Hi-Di formamide (Life Technologies), and sequenced on the automated ABI 3130 Genetic Analyzer.

Finally, sequences were compared using BioEdit v.7.0.8.0 (17) with standard sequences NG_013357.1 of the *PAX9* gene (http://www.ncbi.nlm.nih.gov/nuccore/262331554) and NG_008121.1 of the *MSX1* gene (http://www.ncbi.nlm.nih.gov/nuccore/NG_008121.1) obtained from the GenBank database. Using this approach, polymorphisms and mutations were identified in the genomic DNA sequence of the *PAX9* and *MSX1* genes and their possible effects on gene expression or the protein sequence were described.

Results

Screening of all 300 subjects revealed a total of eight DNA polymorphisms and mutations (Figs 1 and 2). A novel heterozygous g.9527G>T mutation in *PAX9* was identified in a pair of twins (Z612 and Z613), which prompted us to investigate additional members of that family.

We found a g.9527G>T mutation in a pair of identical twins with oligodontia (samples Z612 and Z613, Fig. 3). Both probands, monozygotic twins Z612 and Z613 (23-yr-old women), were first brought to our attention by their orthodontist. Clinical and radiological examinations revealed that both girls had 15 congenitally missing permanent teeth (Table 2). Findings in the maxillae were the same in both girls: all molars, second premolars, and lateral incisors were missing. Tooth agenesis in the lower jaw was slightly different: in twin Z612, the second and third molars in both

Table 1

*Sequences of primers used for PCR amplification and DNA sequencing of human paired box 9 (*PAX9*) and msh homeobox 1 (*MSX1*) exons*

Gene	Exon	PCR amplification		Sequencing reaction
		Forward primer	Reverse primer	
PAX9	1	5′-cagaaagtaatgttagggtcacg-3′	5′-caagtgacagccagaagctc-3′	5′-tgcttatatgctcggaaaaac-3′
	2	5′-ctcccacctatagccttaacttc-3′	5′-agctcccttctcttaaaatcaga-3′	5′-tcgagtcattcacattcaga-3′
	3	5′-gattggacagtgacggtttg-3′	5′-ggaaagacagtgtccctgag-3′	5′-ggacagccccagtagttagt-3′
MSX1	1	5′-gggaacacagaaagatagagacc-3′	5′-ctgggttctggctactcactg-3′	5′-cgccttattagcaagttctc-3′
	2	5′-ttcatttgcaaaaagtagacagg-3′	5′-agagtactaagggactcttccag-3′	5′-cggcactcaatatctggta-3′

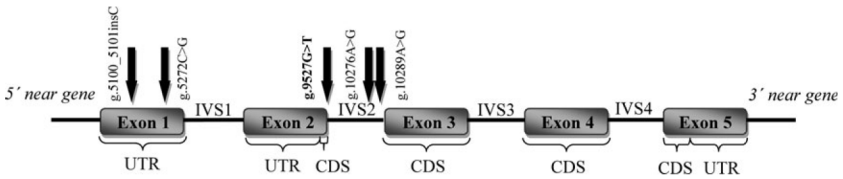

Fig. 1. Localization of mutations and polymorphisms in the paired box 9 (*PAX9*) gene detected in our study. CDS, coding sequence; UTR, untranslated region.

lower quadrants were absent together with the central incisor in the right lower quadrant (Fig. 4). In twin Z613, the second and third molars in both lower quadrants, and the first molar in the lower right quadrant, were missing (Fig. 5). Examination for other ectodermal abnormalities of hair, nails, skin, and history of sweating did not reveal any defects.

Other family members of this twin pair were contacted and asked to participate in family study. The parents of the probands could not be investigated because the mother is no longer living and the father could not be reached. The family history taken at the time of enrollment suggested that the probands' father had a similar dentition developmental disorder. All other family members provided buccal swabs for DNA isolation and underwent clinical examinations. A pedigree construction of the studied family is shown in Fig. 6.

We found a similar pattern in the pedigree in the paternal grandmother (Z613D), aunt (Z613E), and uncle (Z613G) (Fig. 6). Figure 7 shows a radiograph of the unaffected uncle (613H). In the affected uncle (Z613G), the clinical picture differed in both jaws. In the maxilla, agenesis of the second premolars and molars was found, whereas the incisor area was not affected. In the mandible, apart from the missing third and second molars, the second premolars were also absent. The paternal grandmother (Z613D) had all previously existing teeth extracted, and in the aunt (Z613E), the remaining teeth in the maxilla were fitted with crowns, preventing their accurate identification. However, all affected individuals had smaller mesiodistal dimensions of permanent teeth, although the root shape and length appeared to be normal, and a reduction in the size of the crowns and roots in the permanent canines could be observed. The unaffected members of the family pedigree had a normal dentition with a normal (i.e. not reduced) mesiodistal tooth width.

In summary, the same g.9527G>T mutation was found only in the family members with oligodontia (613D, 613E, and 613G, in addition to Z612 and Z613, namely the twins); this mutation was not present in the family members with a normal dentition (613F, 613H, 613I, and 613J) (Fig. 3). The sums of all missing teeth and mutations of the investigated family are given in Tables 2–5.

None of the seven remaining polymorphisms and mutations was associated with tooth agenesis in our screening study. During DNA sequencing we indentified the heterozygous/homozygous insertion of a cytosine at the nucleotide position g.5100_5101insC (rs11373281) in exon 1 of the *PAX9* gene. The position of the insertion could not be accurately determined because of the presence of two adjacent cytosines. The rs11373281 polymorphism occurred both in patients and in subjects with complete dentition. The heterozygous substitution, g.5272C>G (rs4904155), occurred simultaneously with the heterozygous insertion,

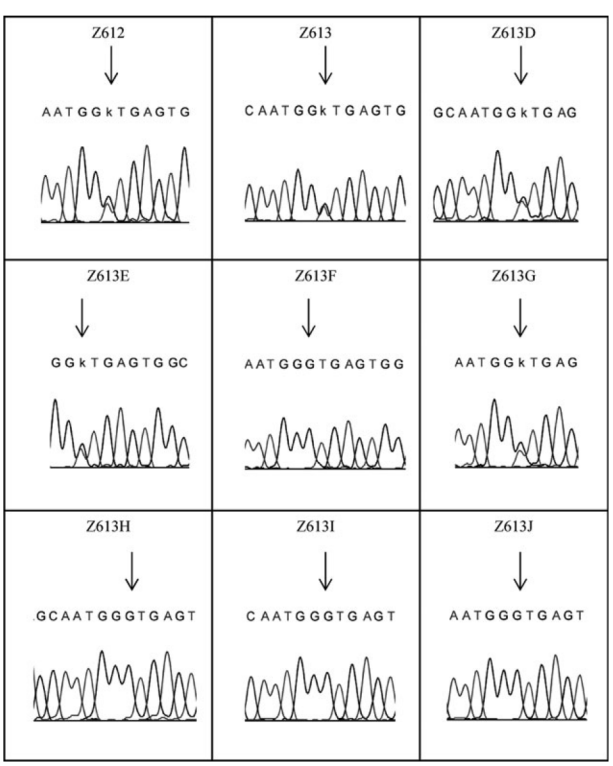

Fig. 3. Chromatograms obtained from DNA sequencing of exon 2 and intron 2 (IVS2) in the paired box 9 (*PAX9*) gene. In samples Z612, Z613, 613D, 613E, and 613G, the g.9527G>T mutation is indicated (k), whereas in samples 613F, 613H, 613 I, and 613J G, a nucleotide in position 9527 is indicated.

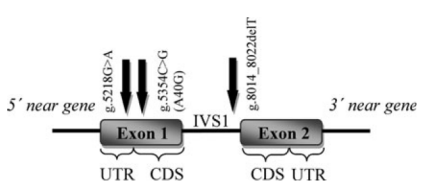

Fig. 2. Localization of mutations and polymorphisms in the msh homeobox 1 (*MSX1*) gene detected in our study. CDS, coding sequence; UTR, untranslated region.

Table 2

Summary of missing teeth in the family with the g.9527G>T mutation

		Quadrant																
		Right								Left								
	Jaw	M			P		C	I		I		C	P		M			
	Upper	18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28	
Family member	Lower	48	47	46	45	44	43	42	41	31	32	33	34	35	36	37	38	Total†
II:1 Z613E	Upper	?	?	?	?			*			*				?	?	?	10 (14)
	Lower	?	?	?											?	?	?	
II:2 Z613D	Upper	?	?	?				?			?			?	?	?	?	11 (15)
	Lower	?	?	?											?	?	?	
III:1 Z613H	Upper																	0 (0)
	Lower																	
III:2 Z613G	Upper	*	*	*	*									*	*	*	*	10 (14)
	Lower	*	*		*									*		*	*	
III:3 Z613I	Upper																	0 (0)
	Lower																	
III:4 Z613F	Upper	*															*	4 (4)
	Lower	*															*	
IV:1 Z613J	Upper																	0 (0)
	Lower																	
IV:2 Z612	Upper	*	*	*	*			*			*			*	*	*	*	11 (15)
	Lower	*	*						*							*	*	
IV:3 Z613	Upper	*	*	*	*			*			*			*	*	*	*	11 (15)
	Lower	*	*	*												*	*	

C, canines; I, incisors; M, molars; P, premolars.
*Missing teeth, and question marks indicate presumably missing teeth.
†Values are presented as the sum of missing teeth (and missing teeth plus third molars are given in parentheses).

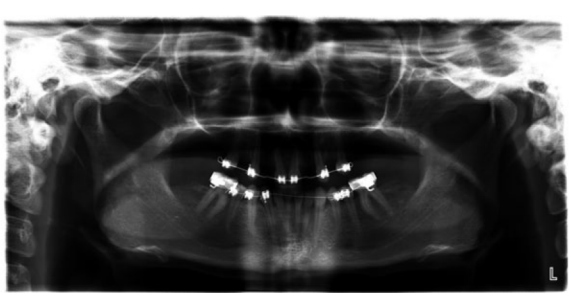

Fig. 4. Panoramic radiograph of monozygotic co-twin Z612 (agenesis 18, 17, 16, 15, 12, 22, 25, 26, 27, 28, 38, 37, 41, 47, and 48; persistent temporary incisor 81).

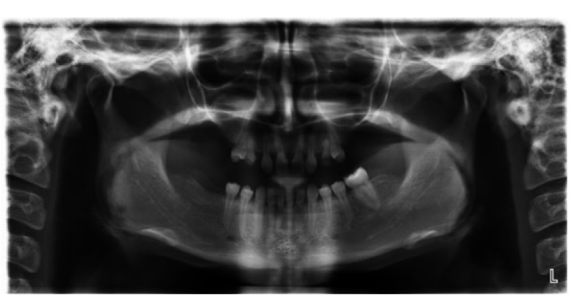

Fig. 5. Panoramic radiograph of monozygotic co-twin Z613 (agenesis 18, 17, 16, 15, 12, 22, 25, 26, 27, 28, 38, 37, 46, 47, and 48; persistent temporary molars 54 and 64).

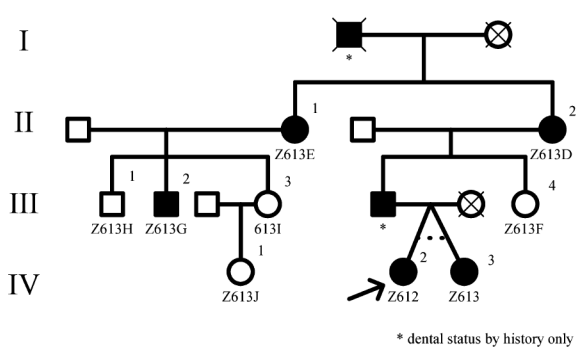

Fig. 6. Pedigree of a multigeneration family with oligodontia. The same g.9527G>T mutation was observed in the family members with oligodontia (Z612, Z613, Z613D, Z613E, and Z613G) but not in genotyped unaffected relatives (Z613H, Z613J, Z613F, and Z613I). The probands' father's status was provided by the family history only. The probands' parents were unavailable for genotyping.

g.5100_5102insC, noted above in samples Z613E, Z613D, Z613J, Z612, and Z613, and the combination of the homozygous insertion, g.5100_5101insC, occurred simultaneously with the homozygous substitution at position g.5272C>G in sample Z613G. These two mutations were always present together and always in the same allelic version.

The polymorphisms g.10276 A>G (rs12882923) and g.10289A>G (rs12883049) were the most common variants found in patients and subjects with complete dentition. Both rs12882923 and rs12883049 polymorphisms can be found in the DNA sequence of intron 2 (before exon 3) in the IVS2 region at positions distant, 54 and 41 bp, respectively, from the first nucleotide of exon 3.

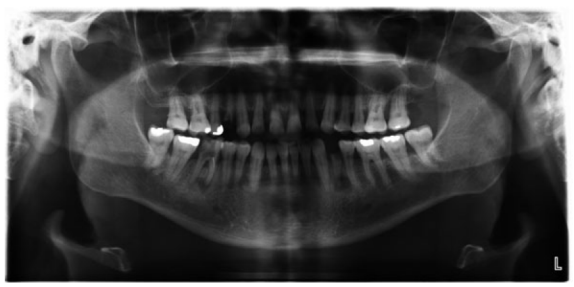

Fig. 7. Panoramic radiograph of the unaffected uncle proband, Z613H (full dentition, condition after tooth extraction 18, 28).

To summarize, we found two polymorphisms in the 5′ untranslated region (5′-UTR) and two polymorphisms in intron 2 of the *PAX9* gene. These polymorphisms have no impact on the amino-acid sequence of the proteins related to the gene and are not directly involved in the regulation of gene expression.

In the *MSX1* gene, the heterozygous mutation, g.5354C>G (rs36059701), which causes the amino-acid change, A40G, was found (Fig. 2). This mutation occurred in family samples Z613E, Z613D, and Z613F with a simultaneous occurrence of the heterozygous deletion of thymine in IVS1 at position g.8014_8022delT. The specific position could not be determined because of nine thymine residues in a row. This deletion has no predicted effect on amino-acid change as it occurs in intron 1; in the homozygous form it was also present in our set of subjects from the screening study. Furthermore, the heterozygous substitution, g.5218G>A (rs186861426), was found in sample Z613J, which is present in the UTR region of exon 1. All these *MSX1* gene mutations occurred in various combinations both in patients and in healthy subjects.

Discussion

Odontogenesis is a complex mechanism regulated by sequential and reciprocal epithelial–mesenchymal interactions, controlled by morphogenetic factors. Several genes have been identified as being expressed during odontogenesis and it is known that mutations in *PAX9*, *MSX1*, axin 2 (*AXIN2*), ectodysplasin A (*EDA*), sprouty homolog 2 (Drosophila) (*SPRY2*), transforming growth factor, alpha (*TGFA*), sprouty homolog 4 (Drosophila) (*SPRY4*), and wingless-type MMTV integration site family, member 10A (*WNT10A*) genes are involved in several forms of tooth agenesis, including syndromes (18). In our study we focused on the analysis of the *PAX9* and *MSX1* genes. Mutation screening was performed in 270 subjects with tooth agenesis and in 30 healthy subjects. The presence of polymorphisms and mutations in affected subjects and healthy subjects was compared and the variants occurring in both groups were excluded as possible factors leading to tooth agenesis.

Table 3

*Mutations found in paired box 9 (*PAX9*) and msh homeobox 1 (*MSX1*) genes in the studied family*

	PAX9		*MSX1*	
Family member	Exon 1	IVS2 (intron 2)	Exon 1	IVS1 (intron 1)
II:1Z613E	g.5100_5101insC, g.5272C>G	**g.9527G > T**g.10276A>G	g.5354C>G (A40G)	g.8014_8022delT
II:2Z613D	g.5100_5101insC, g.5272C>G	**g.9527G > T**g.10276A>G	g.5354C>G (A40G)	g.8014_8022delT
III:1Z613H	Standard seq.*	Standard seq.	Standard seq.*	Standard seq.*
III:2Z613G	g.5100_5101insC, g.5272C>G (homozygotic)	**g.9527G > T**g.10276A>G	Standard seq.*	Standard seq.*
III:3Z613I	Standard seq.*	Standard seq.*	Standard seq.*	Standard seq.*
III:4Z613F	Standard seq.*	Standard seq.*	g.5354C>G (A40G)	g.8014_8022delT
IV:1Z613J	g.5100_5101insC, g.5272C>G	g.10276A>G	g.5218G>A	Standard seq.*
IV:2Z612	g.5100_5101insC, g.5272C>G	**g.9527G > T**, g.10276A>G(homozygotic)g.10289A>G	Standard seq.*	Standard seq.*
IV:3Z613	g.5100_5101insC, g.5272C>G	**g.9527G > T**, g.10276A>G(homozygotic)g.10289A>G	Standard seq.*	Standard seq.*

seq., sequence.
Newly identified mutation is highlighted bold.
*Sequences fully identical with the reference sequences described in the Material and methods.

Table 4

*Description of paired box 9 (*PAX9*) mutations and polymorphisms described in the studied family and their comparison with the corresponding DNA sequences in the GenBank database*

dbSNP rs# cluster id*	Position in the whole gene region†	Exon position		CDS position	Amino acid change‡
rs11373281	g.5100_5101insC	100_101insC	Exon 1	-/5′-UTR	–
rs4904155	g.5272C>G	272C>G		-/5′-UTR	–
None	g.9527G>T	+1G>T	IVS2	-/intron	–
rs12882923	g.10276A>G	-54A>G		-/intron	–
rs12883049	g.10289A>G	-41A>G		-/intron	–

CDS, coding sequence; UTR, untranslated region.
*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).
†According to NCBI Reference Sequence: NG_013357.1 (http://www.ncbi.nlm.nih.gov/nuccore/262331554).
‡According to NCBI CCDS Database: CCDS9662.1 (http://www.ncbi.nlm.nih.gov/CCDS/CcdsBrowse.cgi?REQUEST=CCDS&DATA=CCDS9662.1).

Table 5

*Description of msh homeobox 1 (*MSX1*) mutations and polymorphisms described in the studied family and their comparison with the corresponding DNA sequences in the GenBank database*

dbSNP rs# cluster id*	Position in the whole gene region†	Exon position		CDS position	Amino acid change‡
rs36059701	g.5354C>G	354C>G	Exon 1	c.119C>G	Ala40Gly
rs186861426	g.5218G>A	218G>A		-/5′-UTR	–
none	g.8014–8022 delT	-15_-23delT	IVS1	-/intron	–

CDS, coding sequence; UTR, untranslated region.
*According to NCBI dbSNP Short Genetic Variations: id 4487 (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?showRare=on&chooseRs=all&go=Go&locusId=4487).
†According to NCBI Reference Sequence: NG_008121.1 (http://www.ncbi.nlm.nih.gov/nuccore/NG_008121.1).
‡According to NCBI CCDS Database: CCDS3378.2 (http://www.ncbi.nlm.nih.gov/CCDS/CcdsBrowse.cgi?REQUEST=CCDS&DATA=CCDS3378.2).

The heterozygous g.9527G>T mutation in the *PAX9* gene was detected in two 23-year-old female monozygotic twins. The existence of this mutation had never previously been reported and this is the first time that it was correlated with a specific dental defect. The same g.9527G>T mutation was also observed in three other family members with oligodontia, whereas this mutation was not present in four family members with normal dentition. In this study, all family members with the *PAX9* gene mutation had smaller mesio-distal dimensions of permanent teeth, although the root shape and length were normal.

A further finding in this PAX9 family is that different teeth were affected to different extents. Brook *et al.* (19) reported the greatest reduction in size of permanent canines in hypodontia, in association with the *PAX9* gene mutation. Earlier, mutations in the *PAX9* gene had been identified in association with oligodontia in a family affected with agenesis of most of the permanent molars and a variable absence of second premolars and mandibular incisors (20). Patients with *PAX9* gene mutations have not been reported to have an increased risk of syndromic diseases or abnormalities other than oligodontia (21). In addition, Pawlowska *et al.* (22) sequenced fragments of the *PAX9* gene in 38 individuals with different types of tooth agenesis and in 100 controls. They associated the homozygous g.10276A>G, g.10289A>G, and g.10221G>C mutations with sporadic non-syndromic oligodontia, which is characterized by a congenital lack of six or more teeth (excluding the third molars) that did not occur in any other family member. The present study, on a Czech population, did not confirm this finding. We identified homozygous g.10276A>G and g.10289A>G mutations both in the group of patients and in subjects with complete dentition (11).

The newly identified g.9527G>T mutation is located in intron 2, in the area recognized as the splicing site between exon 2 and intron 2. It is well known that intron splicing is strictly regulated. Enzymes involved in this process are highly specific. After transcription of pre-mRNA begins, the 5′ end of eukaryotic pre-mRNA is capped, the 3′ end is polyadenylated, the introns are cleaved and typical messenger RNA (mRNA)' formed. The left end of the intron (5′ splice site) must incorporate the consensus sequence GU and the right end of the intron (3′ splice site) must include AG bases, the so-called GT-AG rule. The branch site (cryptic site) is necessary for identification of the 3′ splice site. These are three main nucleotide motifs important for successful pre-mRNA splicing (23, 24). About 99% of mammalian genes contain canonical GT-AG dinucleotides for donor and acceptor sites. There are also some minor, non-canonical, GC-AG and AT-AC splice sites

that need special processing (25). These processes may be negatively affected by the g.9527G>T mutation. This mutation, in intron 2 of the *PAX9* gene, contains dinucleotide TT (UU) instead of GT (GU) at the left end, which presumably interferes with correct pre-mRNA splicing as a result of purine to pyrimidine substitution. The error in the pre-mRNA splicing might be responsible for reduced PAX9 production, leading to the development of tooth agenesis in the affected probands investigated in this study.

PAX9 is expressed during embryogenesis, and it has a critical role in tooth development. However, in most tissues, expression of the *PAX9* gene is downregulated after completion of organogenesis and the encoded protein is not detectable in adult organs (26, 27). For this reason, it is not possible to detect expression of *PAX9* in blood samples or buccal swabs and consequently we cannot verify the newly revealed mutation in our patients by cDNA sequencing.

In summary, we identified a novel g.9527G>T mutation associated with oligodontia in a family comprising nine members. We presume that this mutation, located in the region necessary for pre-mRNA splicing, may have led to lower expression of PAX9 protein in the affected probands during dentition development and could have caused tooth agenesis in the family investigated in this study.

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Příloha VII – Next generation sequencing reveals a novel nonsense mutation in *MSX1* gene related to oligodontia

PLOS ONE

RESEARCH ARTICLE

Next generation sequencing reveals a novel nonsense mutation in *MSX1* gene related to oligodontia

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Abstract

Tooth agenesis is one of the most common craniofacial disorders in humans. More than 350 genes have been associated with teeth development. In this study, we enrolled 60 child patients (age 13 to 17) with various types of tooth agenesis. Whole gene sequences of *PAX9*, *MSX1*, *AXIN2*, *EDA*, *EDAR* and *WNT10a* genes were sequenced by next generation sequencing on the Illumina MiSeq platform. We found previously undescribed heterozygous nonsense mutation g.8177G>T (c.610G>T) in *MSX1* gene in one child. Mutation was verified by Sanger sequencing. Sequencing analysis was performed in other family members of the affected child. All family members carrying g.8177G>T mutation suffered from oligodontia (missing more than 6 teeth excluding third molars). Mutation g.8177G>T leads to a stop codon (p.E204X) and premature termination of Msx1 protein translation. Based on previous *in vitro* experiments on mutation disrupting function of Msx1 homeodomain, we assume that the heterozygous g.8177G>T nonsense mutation affects the amount and function of Msx1 protein and leads to tooth agenesis.

Introduction

Tooth development (odontogenesis) is a highly complex process involving interplay between the oral ectoderm and the ectomesenchyme cells putatively derived from the neural crest [1]. Signals from the underlying signalling centres are mediated by more than 350 signalling molecules including transcription factors, growth factors, receptors and other regulation proteins [2]. Synergistic and antagonistic interactions between various peptides and protein molecules may lead to their local activation or inhibition. The final morphology, position and the number

of teeth is fully and precisely determined by these complex signalling networks. It is crucial for the participating molecules to be in the right place at the right time and in an exact quantity [3,4]. Molecular biology methods, such as genome-wide gene expression analysis by microarray assays, revealed expression profiles of the developing dentition and have identified several genes with hitherto unknown functions as possible contributors to the mechanisms of tooth development [5]. The cost and speed of DNA and RNA sequencing have been significantly improved because of the invention of the new sequencing platforms and their continuous iterations to achieve higher efficiency [6]. Next Generation Sequencing (NGS) is one of the major platforms making the recent advances possible [7,8].

Congenitally missing teeth (CMT), also known as tooth/dental agenesis ("hypodontia" is not an accurate term in this context; see below) is one of the most prevalent multifactorial craniofacial defects found in 0.15% - 16.2% of population with a 1.37 times higher prevalence in females than in males [9,10]. According to the number of missing teeth, the dental agenesis can be classified as hypodontia (less than 6 missing teeth other than third molars), oligodontia (6 and more missing teeth other than third molars) while the complete absence of teeth is called anodontia [10].

Congenitally missing teeth can occur in an isolated form or in a relationship with some syndromes such as cleft lip, cleft palate (or both) [11], Down-, Rieger- and Book- syndromes [12], Witkop syndrome associated with *MSX1* gene mutations [13] or ectodermal dysplasia with the most common X-linked inheritance are caused by mutations in *EDA* gene (ectodysplasin), *EDAR* gene (ectodysplasin A-receptor) or *EDARADD* gene (*EDAR* associated death domain) [14]. The *MSX1* gene mutations are typified by autosomal-dominant isolated form of congenitally missing teeth but sporadic forms of congenitally missing teeth are also common variants (see Table 1). The sporadic forms are characterised by the presence of congenitally missing teeth either in a single proband and no other family member or in several members of a family but with no clearly defined pattern of inheritance.

The *MSX1* gene is expressed in dental mesenchyme and plays an important role during reciprocal epithelial-mesenchymal interactions. The Msx1^{-}/ Msx1^{-} deficient mice exhibit complete secondary cleft lip palate and abnormality in tooth development. The development of the tooth is arrested at the bud stage [37,38].

In humans, *MSX1* gene is located on the short arm of chromosome 4 (4p16.2). The structure of the gene consists of 2 exons. Both exons represent UTR´s regions (5´- and 3´- end) and contain 912 bases (including the stop codon) encoding 303 amino acids constituting the human Msx1 protein. The most interesting protein element–the homeodomain—is encoded in the second exon (Fig 1).

The aim of the present study was to screen *PAX9*, *MSX1*, *AXIN2*, *EDA*, *EDAR and WNT10a* genes, using the technique of whole gene DNA sequencing and DNA collected from a group of child patients with oligodontia, in the hope of identifying a mutation which could constitute a possible genetic basis of the patients' phenotype. This resulted in the identification of a novel SNP in the *MSX1* gene as the most likely genetic correlate of the oligodontia. Based on our previous experience with this type of approach [16], we then opted for a family study to further test the relationship between the mutation and the phenotype.

Materials and methods

For the screening of DNA mutations by next generation sequencing (NGS); a total of 60 DNA samples of patients aged between 13 and 17 years with various types of tooth agenesis were obtained at the Clinic of Stomatology of St. Anne's Faculty Hospital in Brno and at the Institute of Dentistry and Oral Sciences in Olomouc.

Table 1. Summary of published mutations in MSX1 gene.

dbSNP rs# cluster id[1]	CDS[2]	Exon no.	Amino acid change[2]	Reference	Phenotype, notice[2]
None	c.62dupG	1	p.G22RfsX168	[15]	Autosomal dominant, non-syndromic, oligodontia
rs36059701	c.119C>G		p.A40G	[16,17]	No significant association
rs121913130	c.182T>A		p.M61K	[18]	Autosomal dominant tooth agenesis*
rs104893852	c.314C>A		p.S105X	[19]	Autosomal dominant tooth agenesis and orofacial clefting*
None	c.416G>A		p.W139X	[20]	Autosomal dominant, non-syndromic oligodontia*
None	c.453G>T	2	p.R151S	[21]	Autosomal dominant hypodontia*
None	c.476T>G		p.L159R	[22]	Autosomal dominant, non-syndromic, oligodontia
None	c.521C>T		p.T174I	[23]	Sporadic form of hypodontia*
None	c.526C>T		p.R176W	[24]	Autosomal dominant, non-syndromic, oligodontia
None	c.559C>T		p.Q187X	[25]	Autosomal dominant, non-syndromic, oligodontia*
None	c.565C>T		p.Q189X	[26]	Oligodontia, cleft lip*
None	c.572_573ins GCAAGTT		p.F191fs	[27]	Autosomal dominant, non-syndromic oligodontia
None	c.581C>T		p.A194V	[28]	Selective non-syndromic oligodontia*
None	c.587G>C		p.R196P	[29]	Autosomal dominant tooth agenesis*
None	c.605C>A		p.S202X	[30]	Witkop syndrome, oligodontia*
None	c.610G>T		p.E204X	This study	Non-syndromic oligodontia
None	c.614T>G		p.L205R	[23]	Autosomal dominant, non-syndromic oligodontia*
None	c.644insA		p.Q216QfsX125	[24]	Autosomal dominant, non-syndromic, oligodontia*
None	c.655G>A		p.A219T	[31]	Autosomal recessive oligodontia with dental anomalies*
None	c.662C>A		p.A221E	[32]	Autosomal dominant, non-syndromic, oligodontia*
None	c.665-666insA		p.N222KfsX118	[33]	Autosomal dominant, non-syndromic, oligodontia
None	c.671T>C		p.L224P	[34]	Autosomal dominant, non-syndromic hypodontia*
None	c.707delG		p.K237SfsX2	[33]	Autosomal dominant, non-syndromic, oligodontia
None	c.750_751insACCGGCTGCC		p.F251PfsX92	[35]	Autosomal dominant, non-syndromic hypodontia
rs515726227	c.910_911dupTA		p.X304YextX48	[36]	Autosomal dominant, non-syndromic, oligodontia

[1]According to NCBI dbSNP Short Genetic Variations: geneID: 4487

[2]Data and localizations were obtained from original articles

*Many of the reviewed articles used what would be an incorrect numbering of exons of the MSX1 gene according to the current reference sequences from NCBI database. The "wrong" numbering is marked with asterisk.

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Dental phenotypes were assessed by oral examinations and inspections of dental radiographs obtained by Planmeca ProMax 3D (Planmeca Oy Finland) using the Planmeca Romexis software (version 2.9.2). Complete dentition was defined as 32 permanent teeth (including the third molars). Oral panoramic radiographs (Planmeca ProMax 3D, Planmeca Oy Finland) were taken in the patient with mutation g.8177G>T (c.610G>T) and members of his family (samples Z606B, Z606C, Z606D, Z606E, Z606F). The study protocol and informed consents (No: Fm-L009-001-ZUBNI-014) were reviewed and approved by ethical committees of Faculty of Medicine and Dentistry, Olomouc and Faculty of Medicine, Brno. The study was performed in accordance with the Declaration of Helsinki. All participants in this study gave their written informed consent.

200 μL of whole blood were taken from the fingers by EDTA coated capillaries Microvette 200 μL K3 EDTA (Sarstedt, Germany). DNA was extracted from 200 μL whole blood by using automatic instrument ZEPHYRUS Magneto with modified protocol as specified for BodyFluid DNA/RNA isolation kit (Elisabeth Pharmacon, Czech Republic). The yield of genomic DNA was verified by spectrophotometric measurement. Size and quantity of DNA fragments were

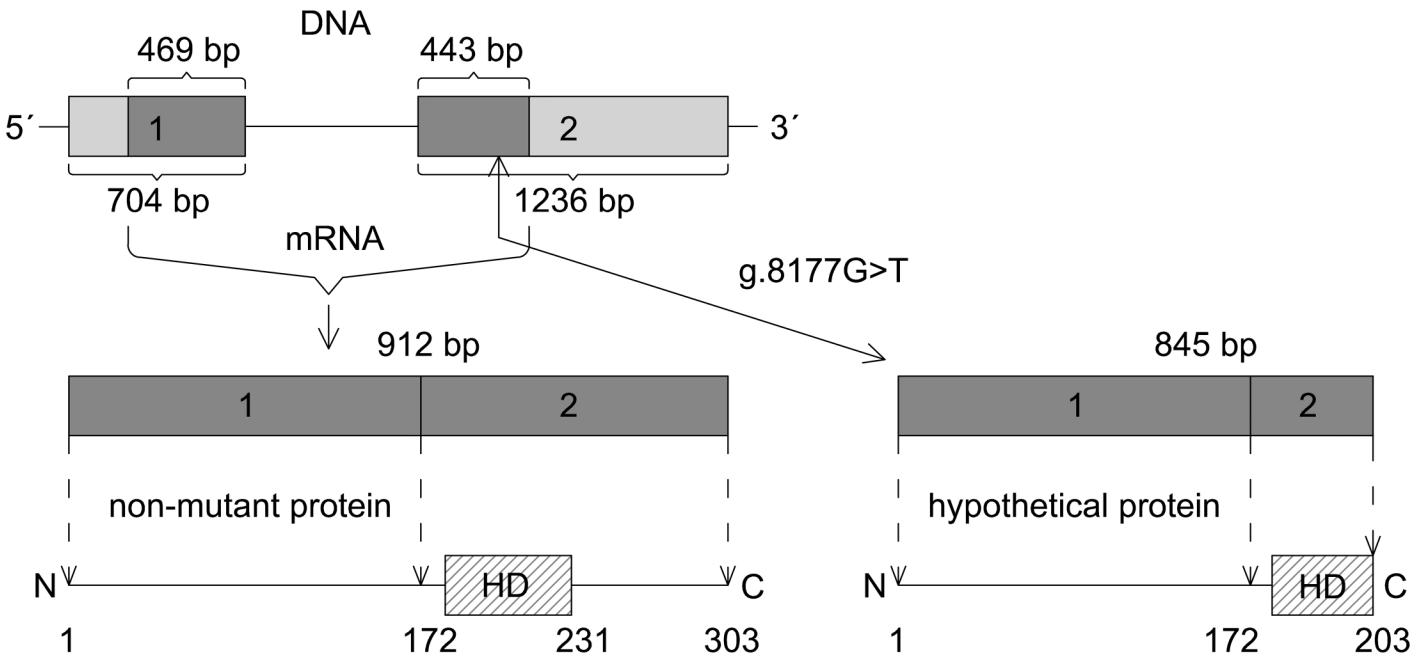

Fig 1. Schematic description of nucleotide sequence, mRNA and protein structure of the MSX1. Light grey colour shows untranslated region (UTR); dark grey colour shows coding sequence (CDS); HD = homeodomain. The hypothetical mutated protein product could be 100 amino acids shorter (203 amino acids) than non-mutant variant of the Msx1 (303 amino acids).

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determined on 2200 TapeStation Instrument (Agilent Technologies, USA). Paired end sequencing libraries were constructed from 150 ng of fragmented DNA in 50 μL PCR water using the Covaris S220 Sonicator (Covaris, USA) to a size 200 bp (treatment time 180 s, volume 130 μL). Library preparation was performed by SeqCap EZ System (Roche NimbleGen, USA) with probes for *PAX9*, *MSX1*, *AXIN2*, *EDA*, *EDAR*, and *WNT10A* genes according to manufacturer's recommendations. Twelve samples were mixed to one library. The concentration of captured library was determined through KAPA qPCR KAPA Library Quantification Kit (Kapa Biosystems, USA) to produce clusters suitable for the Illumina MiSeq platform (Illumina, USA). The library was sequenced on a MiSeq V2 2x151 bp sequencing run according to manufacturer recommendations for 14 pM libraries.

Obtained NGS data were analyzed according to Roche NimbleGen workflow [39]. Hg38 was used as the reference genome. BWA 0.7.13 software package [40] was used for indexing the reference genome and alignment of reads. Adapter trimming was performed using Trimmomatic 0.32 software [41]. PCR duplicates were removed using Picard Tools 1.110 software. Variant calling and filtering was performed using SAMtools 1.3 and BCFtools 1.3 software [42,43]. The depth of coverage was calculated for each position on the reference genome corresponding to the selected genes (*PAX9*, *MSX1*, *AXIN2*, *EDA*, *EDAR*, and *WNT10A*) using software R [44]. Sequence data were browsed through using Integrative Genomics Viewer 2.3 (IGV) [45,46].

For control sequencing of the second exon of the *MSX1* gene and intron-exon boundaries, the automated ABI 3130 Genetic Analyzer was used. DNA sequence was amplified by PCR in a 20 μL reaction volume. Specific PCR method and sequencing primers were used as described previously [16].

PCR analysis of exon 2 of the *MSX1* gene was performed by the KAPA2G Robust HotStart kit (Kapa Biosystems, USA) + 5% DMSO and 1% 7-Deaza-dGTP (Roche Diagnostics, USA).

Table 2. Amino-acid sequence for non-mutant and mutant Msx1 homeodomain used for homology modelling.

MSX-1 (residues 173–230)	RKPRTPFTTAQLLALERKFRQKQYLSIAERAEFSSSLSLTETQVKIWFQNRRAKAKRL
MSX-1 mutant (residue 173–203)	RKPRTPFTTAQLLALERKFRQKQYLSIAERA

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Robust polymerase, DMSO and 7-Deaza-dGTP were used for PCR mixtures because of the very high content of G + C bases in the nucleotide structure of *MSX1* gene. The conditions for PCR were 95˚C for 10 min activation/denaturation step, followed by 40 cycles of 95˚C for 60 s, 58˚C (with 20% ramp) for 30 s, 72˚C for 60 s, with a final extension for 7 min. The Veriti thermal cycler (Applied Biosystems, USA) was used for all PCR reactions.

Amplicons were purified by ExoI-FastAP (Fermentas, USA). The mixtures were incubated at 37˚C for 15 min and subsequently at 85˚C for 15 min to inactivate enzymes. Sequencing was performed with BigDye Terminator v.3.1 (Life Technologies, USA). Sequencing reactions/the products were purified by EDTA/ethanol precipitation, resuspended in 10 μL Hi-Di Formamide (Life Technologies, USA), and sequenced on the automated ABI 3130 Genetic Analyzer.

Finally, sequences were compared with the standard sequence NG_008121.1 of the *MSX1* gene obtained from the GenBank database by MEGA 7 software.

Molecular modelling

Both the wild type and truncated version (Table 2) of Msx1 homeodomain (residues 173–230 and 173–203, respectively) were prepared using homology modelling software MODELLER [47] based on PDB template 1IG7. Schematic representation was prepared via visual molecular dynamics software (VMD) [48], Schrodinger Maestro software (Schrodinger, L. Biologics Suite 2017–1) and PyMOL software (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC). DNA fragment was superimposed with the prepared structures from PDB in PyMOL.

Results

Using the technique of whole gene next generation sequencing (NGS), DNA samples collected from 60 child patients were sequenced for mutation in *PAX9*, *MSX1*, *AXIN2*, *EDA*, *EDAR and WNT10a* genes. More than 3000 polymorphisms were found in the screened genes. We then looked for mutations which could be putatively linked to the oligodontia phenotype. In one female proband, we found a mutation in the *MSX1* gene causing premature stop codon in the second exon of the gene thus resulting in a significantly shorter version of the protein. We found no other mutations in the *MSX1* gene in this patient or in the remaining probands in the group. Additional screening of the female proband's DNA, particularly of the genes listed above, revealed no other mutations which would correlate with her phenotype. We then opted for a family study to further test the relationship between the newly identified mutation and the oligodontia phenotype.

The female proband (Z606) displayed oligodontia with 17 missing teeth (including third molars, see Fig 2A). To further characterize her phenotype, we excluded the presence of ectodermal dysplasia, Witkop syndrome (thick hair, normal development of eyebrows, eyelashes, nails–hands and feet—see Fig 2B and 2C), defective development of sweat glands and the presence of cleft lip/palate. In addition, using standard clinical genetic examination, we excluded other hereditary syndromes often present in patients with oligodontia.

Genealogic examination revealed isolated oligodontia in the mother of the proband (Z606B) and in her father (information about father were obtained from family history only). The mother had no knowledge of other cases of oligodontia in the family. Two siblings of the

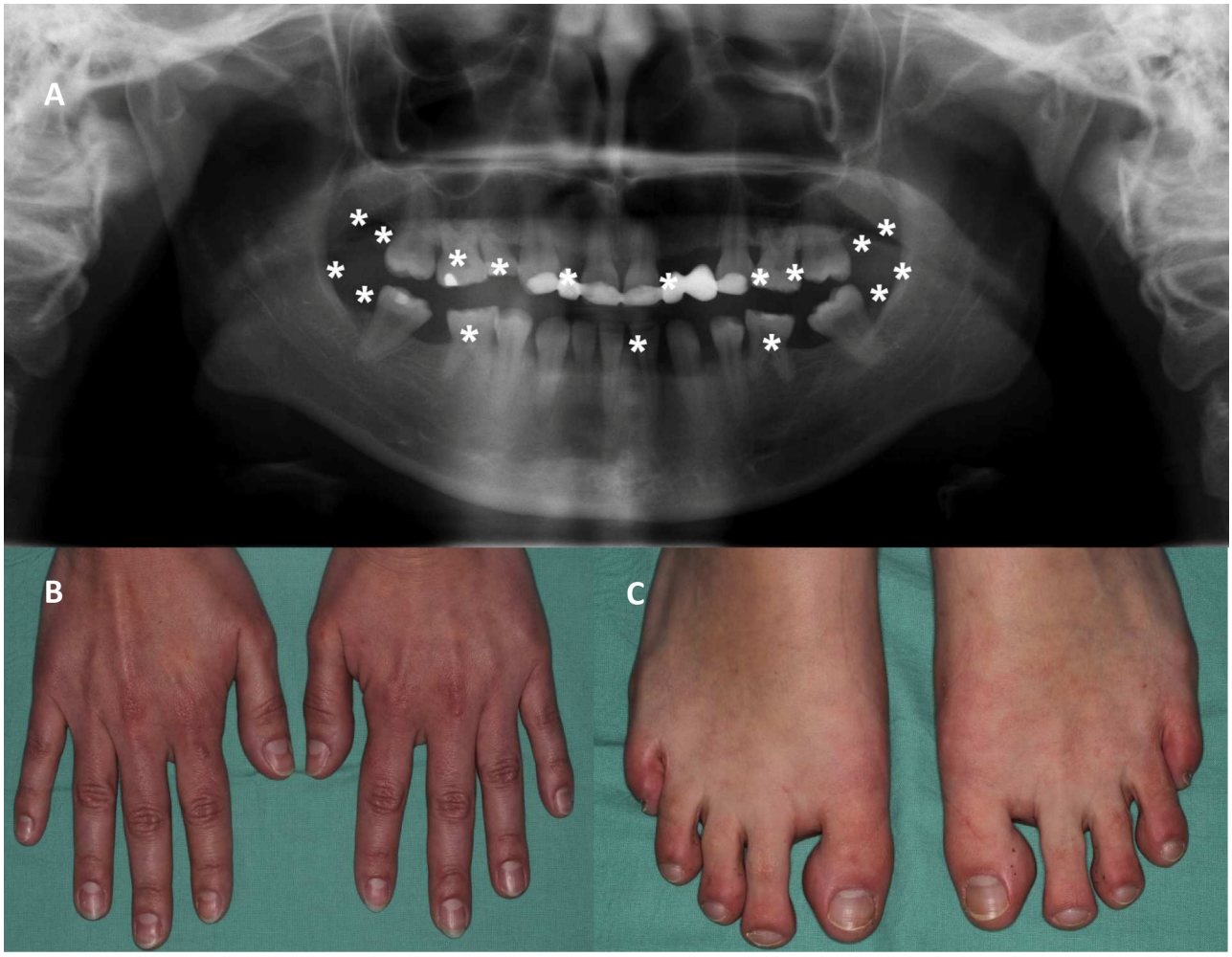

Fig 2. Proband´s tooth and nail phenotype. (A) Panoramic radiograph of proband Z606. This individual has a total 17 congenitally missing teeth (Agenesis 18, 17, 15, 14, 12, 22, 24, 25, 27, 28, 38, 37, 35, 31, 45, 47, 48). Condition after orthodontic and prosthetic treatment. (B) Nails on hands are in normal shape without any defect. (C) Nails on legs are in normal shape and without defects excepts the fifth finger nail that is hypoplastic.

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mother (Z606E and Z606F) are healthy and have healthy children. An elder brother of the proband (Z606D) and the father (Z606C) have healthy developed dentition. The summary of all missing teeth and the reconstruction of the family pedigree are in Table 3 and in Fig 3 respectively.

We then proceeded to collect DNA from five family members of Z606 proband and subjected the samples to capillary sequencing focusing on the second exon of the *MSX1* gene.

The mutation which we found earlier in Z606 and for which we were looking in her relatives, has not, to our knowledge, been previously reported in the literature. The heterozygous nucleotide change was identified in the second exon of the *MSX1*gene at the position g.8177G>T, using NG_008121.1 as the reference sequence (GenBank Database). The total average depth of coverage of analyzed sample was 98 and the mutated position was read 49 times; 17 times (35%) for G allele and 32 times (65%) for T allele. The nucleotide change produces the premature stop codon at the amino acid position 204 that would otherwise code for glutamic acid (p.204E -> p.204Stop). The triplet GAG is changed to TAG, or, in the case of mRNA, to UAG. The mutated protein product is 100 amino acids shorter than the non-

Table 3. Summary of missing teeth in family with g.8177G>T nonsense mutation. Asterisks indicate the missing teeth. The column shows the sum of missing teeth, in parentheses missing teeth plus third molars are given (I. . .incisors, C. . .canines, P. . .premolars, M. . .molars).

Right quadrant									Left quadrant								
	M			P		C		I	I	C		P		M			
Upper	18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28	Total
Lower	48	47	46	45	44	43	42	41	31	32	33	34	35	36	37	38	
II:1 Z606E	*																0(1)
II:2 Z606F																	0(0)
II:3 Z606C																	0(0)
II:4 Z606B	*			*	*		*			*		*	*			*	9(13)
	*			*				*					*			*	
III:1 Z606D																	0(0)
III:2 Z606	*	*		*	*		*			*		*	*		*	*	13(17)
	*	*		*					*				*		*	*	

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mutant variant of the *MSX1*. The structures of both non-mutant and mutant variants of *MSX1* are shown in Fig 1.

We found the same heterozygous mutation g.8177G>T in one other family member (Z606B) with oligodontia. Mutation was not present in other family members who had complete sets of teeth (Z606C, Z606D, Z606E and Z606F). One additional mutation, g.8014_8022delT, was identified in the second exon of the *MSX1* gene in a sample collected from Z606F. This deletion was not associated with tooth agenesis [16]. This sample was further re-sequenced by a reverse primer normally intended for PCR amplification, the reason being that the deletion renders the use of the standard sequence primer (forward reading) unsuitable; indeed, its application lead to an unreadable chromatogram. The chromatograms from capillary sequencing are shown in Fig 4. No additional mutations were detected in *PAX9*, *AXIN2*, *EDA*, *EDAR* or *WNT10a* genes.

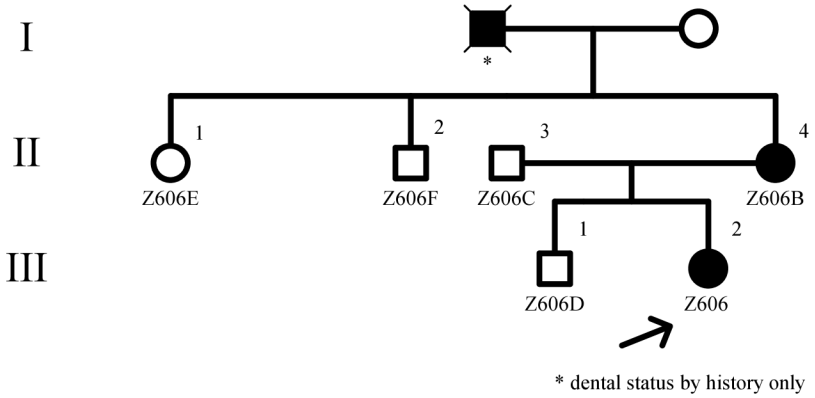

Fig 3. Pedigree of multigeneration family with oligodontia. The same g. 8177G>T mutation was observed only in family members with oligodontia (Z606 and Z606B) but not in unaffected relatives (Z606C, Z606D, Z606E, Z606F). The probands' grandfather's status was provided by family history only. The probands' grandmother was unavailable for genotyping.

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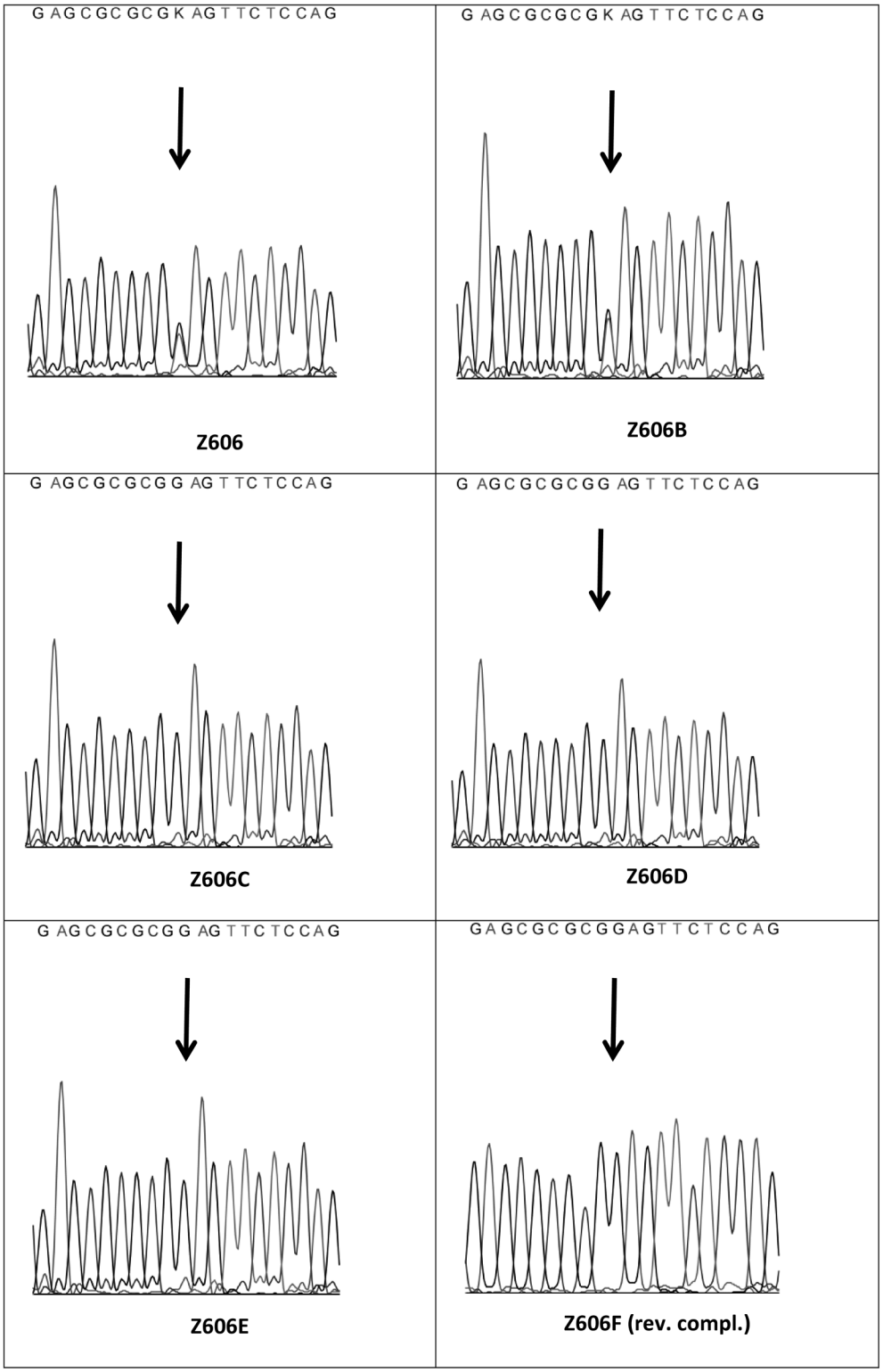

Fig 4. Chromatograms obtained from capillary sequencing of exon 2 in MSX1 gene. In samples Z606 and 606B is g.8177G>T mutation marked as "K" mixed base (G/T), in samples 606C, 606D, 606E, 606F G nucleotide in the 9527th position of the MSX1 gene is indicated. In case of sample Z606F is chromatogram reverse complement.

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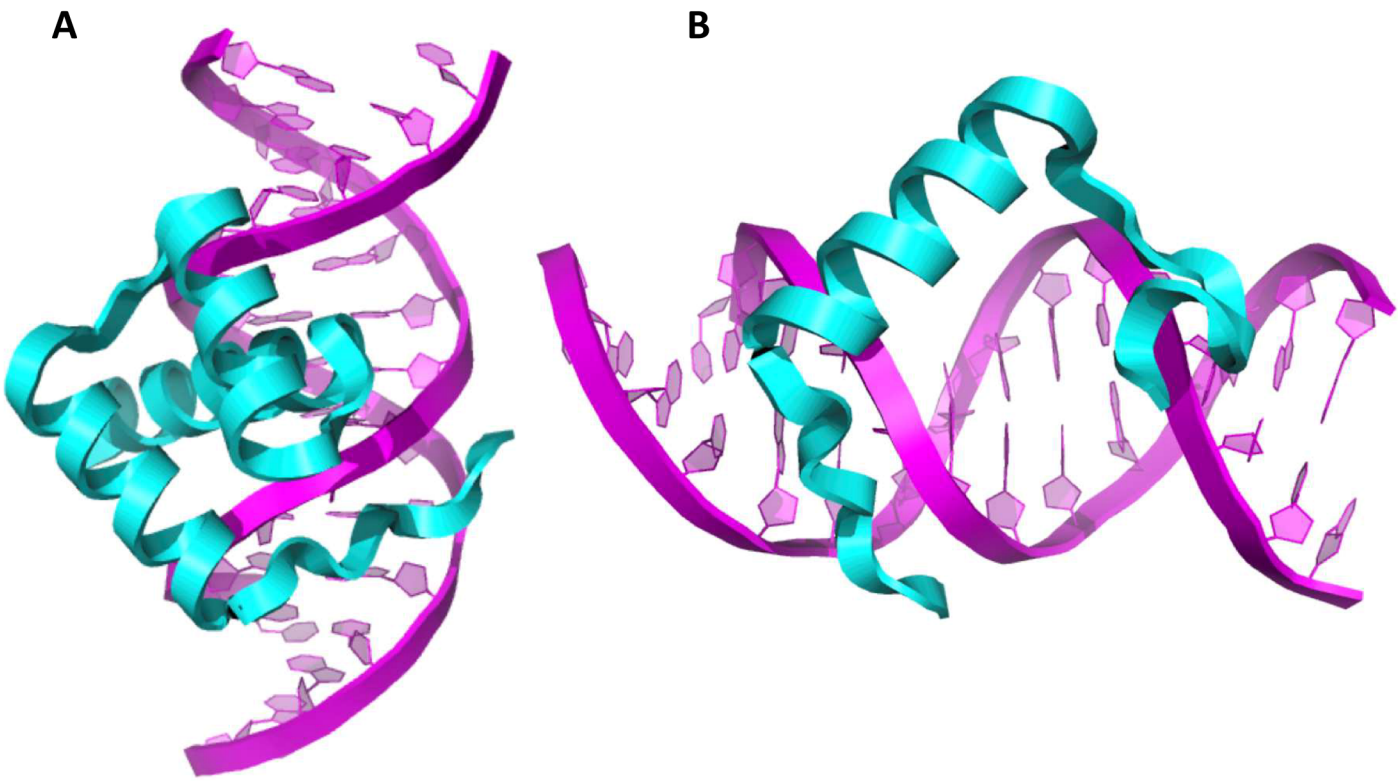

Fig 5. Msx1 homeodomains bound to DNA. (A) Non-mutant Msx1 homeodomain. (B) Mutant Msx1 homeodomain. Homeodomain is visualized as cartoon.

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Discussion

To date, over 30 mutations in *MSX1* gene have been related to tooth developmental diseases (see in Table 1). *MSX1* gene together with *PAX9* gene (recently reviewed by Bonczek *et al*, 2017 [49]) are two most studied genes related to tooth agenesis.

The heterozygous g.8177G>T substitution, identified for the first time in the present study, leads to premature stop codon and results in a protein sequence truncated to 203 amino acids compared to the normal protein with 303 amino acids. This novel mutation was identified neither in our previous capillary sequencing project screening 270 Czech individuals with tooth agenesis and 30 unrelated healthy control subjects [16] nor, to the best of our knowledge, in any other studies. There have been five other reports, though, of substitutions, each of them resulting in an appearance of the premature stop codon [19,20,25,26,30] ("see Table 1").

Typically, the mutations in *MSX1* gene are associated with oligodontia inherited in the autosomal dominant manner; to date only one case of autosomal recessive oligodontia has appeared [31]. All these mutations are heterozygous and the missing teeth may result from haploinsufficiency of the Msx1 protein [50]. Affected individuals are missing a large number of teeth (mostly more than 10), [18,29,30]. This is also the case of the two family members identified in the present study who are missing third molars, second premolars, upper first molars, upper second incisors and one lower first incisor. The proband Z606 has more severe oligodontia, missing also second molars (together 17 teeth).

Most of the previously reported mutations (Table 1) were located in the highly conserved homeodomain (172^{nd}-231^{st} amino acids) which is thought to play an important role in protein-protein interactions and DNA binding [51]. It has been known that an important factor

in the protein stability is the presence of helix II [52] while helix III is important for DNA binding [53]. A protein-protein interaction between Pax9 and Msx1 has been demonstrated both *in vivo* and *in vitro* and it seems that this interaction is essential for the expression of *BMP4* in the mesenchyme [54]. Furthermore, Msx1 protein forms dimeric complexes with Dlx proteins through their homeo-domains; the binding being mediated by residues that are also required for their DNA binding activities [51]. The newly found heterozygous nonsense nucleotide change g.8177G>T is located in helix II of the homeodomain, thus probably affecting helix II and causing absence of helix III. Thus it seems that the polymorphisms affecting the homeodomain and associated with oligodontias, including the one in the present study, lead to amino acid changes that impact on the protein stability and/or the homeodomain's DNA binding activity. In order to gain greater insight into the mechanism, we modelled the structures of homeodomains of both the non-mutant and mutant version of Msx1 (Fig 5A and 5B). As hypothesized, the structural modelling indeed revealed that the truncated version of Msx1 homeodomain lacks, at least in part, the ability to interact with DNA.

Cases of nucleotide changes leading to premature stop codon and having functional or morphological consequence have been described previously. For example, Liang *et al* (2012) [26] published heterozygous Q189X substitution in a Chinese family suffered from oligodontia and cleft lip. They performed relative-quantitative PCR and found out that mRNA expression level of the mutant *MSX1* was less than half of the non-mutant. Kimura *et al* (2014) [20] identified heterozygous nonsense c.416>A mutation producing W139X protein product in Japanese family with non-syndromic oligodontia. They carried out a functional expression study and reported no difference in the expression level between the mutant and non-mutant variants of Msx1, however, they noted a severe defect in the nuclear trafficking of the mutant protein.

In summary, we have found a heterozygous nonsense g.8177G>T mutation in *MSX1* gene which appears to be associated with severe tooth agenesis. Based on a review of previously published data complemented by the molecular modelling performed in the present study, we conclude that the newly identified mutation could influence specific properties of Msx1 protein thus possibly impairing its ability to interact with DNA and proteins. The mutation may therefore, produce a significant adverse effect on the role of Msx1 protein in the complex signaling mechanisms necessary for normal odontogenesis and result in oligodontia in the affected individuals.

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Project administration: Omar Šerý.

Software: Tomáš Zeman, Tereza Gerguri.

Supervision: Lýdie Izakovičová-Hollá, Jiří Vaněk, Omar Šerý.

Validation: Jana Šoukalová.

Visualization: Tereza Gerguri.

Writing – original draft: Ondřej Bonczek, Peter Bielik.

Writing – review & editing: Vladimir J. Balcar, Omar Šerý.

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