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Exclusion of coding region mutations in *MSX1*, *PAX9* and *AXIN2* in eight patients with severe oligodontia phenotype

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Structured Abstract

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Purpose – This paper describes the screening of eight patients with severe oligodontia for *PAX9* and *AXIN2* mutations.

Subjects and Methods – Anamnestic data and a panoramic radiograph were collected to study the phenotype of eight patients with oligodontia and their first-degree relatives. A blood sample was taken for a mutational screening for *PAX9* and *AXIN2* mutations.

Results – No mutations were discovered, but a unique nucleotide change in a conserved 5′ flanking region of PAX9 was revealed. Earlier screening of the same patients for *MSX1* mutations also had a negative outcome.

Conclusions – Considering the discrepancy between the high incidence rate of agenesis and the relatively small number of reported causative mutations in *PAX9*, *MSX1* and *AXIN2* genes, the genetic contribution to oligodontia probably is much more heterogeneous than expected so far. Therefore negative results, like the present exclusion data, should be published more often in order to get a better appreciation of the relative contribution of these specific mutations causing oligodontia. In this context the exact number of tested probands also should be mentioned at all cases. Recent evidence of PAX9–MSX1 protein interactions in odontogenesis as well as other genes and developmental factors should receive more attention.

Key words: agenesis; *AXIN2*; *MSX1*; odontogenesis; oligodontia; *PAX9*

Introduction

Agenesis of permanent teeth is one of the most common developmental anomalies in humans, with an overall incidence of 1.6–9.6%, if missing of third molars

is excluded (1). European and Australian Caucasian populations appear to be affected more often than North American Caucasians (2). Probably because of better diagnostic screening and more frequent reporting, this incidence of agenesis falsely seems to have increased during the last decades (3). Oligodontia or the absence of six or more teeth is less frequent (0.08–1.1%) (4–6). Most affected teeth are mandibular second premolars, followed by lateral maxillary incisors and the maxillary second premolars (2). A large variation in location, symmetry and amount of agenetic teeth exists (1). Primary teeth are seldom affected (7). Agenesis can appear isolated in a sporadic or familial way, or can be associated with a syndrome (8). The inheritance pattern is autosomal dominant, autosomal recessive or X-linked (1,9). Females seem to be affected slightly more than males (2,3). Different causes are possible for tooth agenesis: environmental factors (6) (e.g. trauma), multi-reagent chemotherapy, radiotherapy (10) or genetic factors (11). So far mutations in three different genes have been identified as a possible cause for isolated agenesis. Mutations of the *MSX1* or the *PAX9* genes create an impairment of one or more molecular processes that regulate tooth formation (12). More recently *AXIN2* mutations have been reported causing colon cancer and agenesis of teeth (13). *PAX9* mutations have been predominantly found in oligodontia patients with missing molars (5,14–21). In most of the affected cases also some other teeth were missing (18,19). Because patients with a similar phenotype were identified in our clinic, it was hypothesized that mutations of *PAX9* or *AXIN2* could be the cause of the severe oligodontia in these cases, as earlier *MSX1* mutation screening was negative. It was the aim of this study to screen these eight patients and their first-degree relatives for *PAX9* and *AXIN2* mutations and to review the different gene mutations causing tooth agenesis so far.

Materials and methods

Eight individuals with oligodontia were selected from a high-risk group of patients who had earlier been screened for *MSX1* mutations in a study by De Muynck et al. (22) at the Centre of Human Genetics of the KU-Leuven. Patients and their parents consented to cooperate in the study which was also approved by the Ethical Committee of the University Hospital of the Catholic University of Leuven. They all signed the informed consent form. A blood sample and a panoramic radiograph were taken from the patients and their first-degree relatives. Anamnestic data of medical history were recorded and a familial anamnesis for the occurrence of agenesis was also performed. The same DNA samples as in the previous study (22) were used, but only from a selected group of patients (≥6 congenitally missing teeth, no cleft lip and palate (CLP) history, occurrence of agenesis in close relatives). Exons and 5′ flanking conserved regions were amplified by polymerase chain reaction (PCR) with DyNAzyme^{TM} EXT DNA polymerase (Finnzymes, Espoo, Finland). PCR primers and conditions for exons were as previously described (13,14) except that for exon 1 of PAX9 an annealing temperature of 61°C was used. For the immediate 5′ flanking region (promoter) of PAX9 primers CACTGGCAATTGGTCGACTT (forward) and CCCACCTGGG TGACTAAATAC (reverse) and annealing temperature of 59°C and 32 cycles were used. For the upstream conserved region of PAX9 (−5157 to −4488 with respect to the translation start codon) primers CGCAACTTCTGCTAATGCTG (forward) and GTCGCCCGCTTTCTCCTT (reverse) and a method with 2 cycles in 57°C, 2 cycles in 55°C and 32 cycles in 53°C were used and PCR reaction was supplemented with 2% DMSO. PCR products were purified enzymatically with ExoSap-IT reagent (USB, Cleveland, OH, USA) according to the manufacturer's instructions. PCR products were sequenced with dye terminator chemistry (ABI Prism® BigDye^{TM} Terminator Cycle sequencing kit, version 2.0; Applied Biosystems, Foster City, CA, USA), and analysed in 4% denaturing gels on the ABI 377 DNA sequencer. Sequencing results were compared by BLAST2 (http://ncbi.nlm.nih.com/gorf/bl2.html) with EMBL entries AJ238381, AJ238382, and AJ238383 or with human genomic contig NT_026437.10.

Results

All eight patients suffered from severe oligodontia. The average number of missing teeth (excluding the wisdom teeth) in this sample was 12.5, ranging from 7 to 24 (Table 1). Normal primary teeth were reported by all individuals and their parents. None of the patients suffered from a syndromic disease. In all cases at least

Table 1. **Patient group**

	Congenitally missing permanent teeth															
	Right								Left							
Individuals ID and dental arch	8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8
LS																
Maxillary	*	*		*									*		*	*
Mandibular	*		*	*								*	*	*	*	*
KS																
Maxillary	*			*	*							*	*			*
Mandibular	*			*	*								*			*
WC																
Maxillary	*	*		*	*		*			*			*	*		
Mandibular				*								*	*			*
KD																
Maxillary						*	*			*	*					
Mandibular					*		*					*				
AB																
Maxillary				*	*	*	*			*	*	*				
Mandibular				*	*								*			*
CA[†]																
Maxillary	*			*	*	*	*			*	*	*	*		*	*
Mandibular		*		*	*	*	*	*	*	*	*	*	*		*	*
RE[‡]																
Maxillary	*	*	*	*	*		*				*	*	*	*	*	*
Mandibular	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
KvN																
Maxillary	*	*	*	*									*	*	*	*
Mandibular	*	*		*					*				*		*	*

*Congenitally missing tooth: 1 and 2 = central and lateral incisors, respectively; 3 = canine; 4 and 5 = first and second premolars, respectively; 6, 7 and 8 = first, second and third molars, respectively.
[†]Unique variation –5084C > A (5′conserved region)
[‡]Rare haplotype with IVS1–82G > A and IVS2–62G > C.

one of the parents also had agenesis of at least one tooth and there was a positive response (sometimes vaguely) to the presence of agenesis in more distant relatives. No clear segregation pattern was however present. The mutation screen did not reveal any mutations in the PAX9 or AXIN2 coding regions or exon–intron junctions. However, in one patient, a heterozygous one nucleotide change C > A was found in the 3′ untranslated region of a mRNA of a hypothetical protein Q96HD6 (gene BC008699). The same patient was homozygous for the exon 3 718G > C (Ala240Pro) polymorphism of PAX9 (14). A rare haplotype containing IVS1-82G > A and IVS2-62G > C was observed in another patient. At first glance this patient was diagnosed to have ectodermal dysplasia. Further clinical examination countered this diagnosis. These single nucleotide polymorphisms (SNPs) seem to be rather unique as they have been tested in our laboratory altogether with 245 samples (other research lines), including 80 unaffected unrelated controls. So far these genotypes were only present in these particular patients. No polymorphisms have been discovered for *AXIN2* and earlier screening in this selected group for *MSX1* mutations also had a negative outcome. The parents of these patients have not been tested as the testing of the probands did not reveal any mutations.

Discussion

PAX9 belongs to a gene family encoding for transcription factors during global embryogenesis. It contains a paired box, a sequence encoding a specific DNA-binding domain. During odontogenesis PAX9 plays an important role in the sequential and reciprocal signalling cascades between epithelial and mesenchymal cell layers. Mice homozygous for *Pax9* deletion lack pharyngeal pouch derivates, develop craniofacial and limb abnormalities and the development of teeth is arrested at the bud stage. Heterozygous *Pax9* mice show no abnormalities (12,23,24).

Up to now 15 heterozygous mutations of the *PAX9* gene have been reported (Fig. 1, Table 2). Fourteen of these were associated with familial, non-syndromic form of tooth agenesis. Although there is considerable phenotypic heterogeneity, molars are the most affected teeth (5,14–21,25–28). However, in our patient group second premolars are the most frequently missing teeth in the lower jaw as well as in the upper jaw, followed by the first premolars, the second molars, the lateral incisors, the canines, the first molars and the central incisors (wisdom teeth excluded) (Table 1). This pattern of tooth agenesis might be indicative for the presence of *MSX1* mutations (29). On the other hand molars, especially second molars, are also frequently missing in these cases, which is an important factor for distinguishing *PAX9* from *MSX1* mutations (29). Prioritizing a candidate gene for mutations based upon the phenotypic pattern of tooth agenesis thus still remains difficult. The resemblance between these phenotypes probably reflects the close interactions of PAX9 and MSX1 during early tooth development. Co-expression during the bud stage seems to be necessary for BMP4 expression, which enables the induction of the enamel knot (30,31).

The *MSX1* gene is expressed in the dental mesenchyme during odontogenesis. As a member of the homeobox family, this gene encodes for a DNA binding sequence. The MSX1 protein represses transcription and, besides PAX9, it also interacts with other components during the signalling pathways of odontogenesis like the DLX-family or TATA-binding protein (TBP) (30–33). Homozygous *Msx1*-deficient mice exhibit craniofacial deformities like secondary cleft palate, deficiencies in mandibular and maxillary alveolar processes and disturbed tooth development during transition from bud to cap stage. Heterozygous mice have no abnormalities (12,34,35).

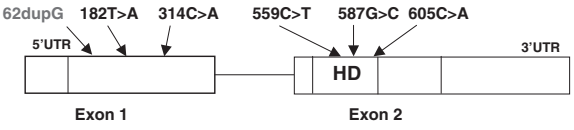

Fig. 1. Schematic view of human PAX9. PD = the region encoding the paired domain; arrows indicate location of known mutations (28).

So far, seven *MSX1* mutations as well as some whole gene deletions have been discovered in oligodontia patients, all heterozygous (Fig. 2, Table 2). There are six cases of familial tooth agenesis; one is associated with tooth agenesis and nail dysplasia. Deletions of MSX1 have been reported in patients with the Wolf-Hirschhorn syndrome. In one Dutch family hypodontia is associated with CLP. Third molars, second premolars and incisors seem to be the most frequently missing teeth (25,29,30,36–41).

The *AXIN2* gene is located on chromosome 17 and is known as a negative feedback regulator of Wnt-signalling, which regulates early organ differentiation and development and plays a key role in many basic cell functions, like cell homeostasis. Disturbance of Wnt-signalling may cause cancer. Experiments in mice have also demonstrated the importance of normal Wnt-signalling for the development of teeth. In mutant mice tooth development can be stopped by blocking the Wnt pathway. During tooth development AXIN2 is expressed in the dental mesenchyme, the odontoblasts and the enamel knot, and it is suggested that it is needed for downregulation of Wnt-signalling at specific stages (13).

Recently two *AXIN2* mutations have been reported (Fig. 3, Table 2) (13). One was reported in a large family with familial tooth agenesis and colorectal cancer or precancerous lesions of variable types. The oligodontia phenotype here is rather severe as the affected family members lacked most permanent molars, premolars, lower incisors and upper lateral incisors. The other was a *de novo* germline mutation in a 13-year-old patient with an oligodontia phenotype as described above. Because of his young age the cancer predisposition could not be demonstrated (13). It is remarkable that a mutation of the *AXIN2* gene seems to cause hypoplasia in one organ (the teeth) and hyperplasia (colorectal cancer) in another (13). Three novel *AXIN2* gene polymorphisms also have been reported only recently (42)

Table 2. **Current overview of the known mutations of PAX9 and MSX1 in patients with tooth agenesis**

Gene	Mutation	Localization	Mutated protein	Phenotype	References
MSX1	587G > C-missense	Exon 2, homeobox domain	Arg196Pro	Autosomal dominant FTA	36
	314C > A-transversion	Exon 1	Ser105Stop	Autosomal dominant FTA and CP/CLP	37
	605C > A-transversion	Exon 2,homeobox domain	Ser202Stop	Witkop syndrome	39
	182T > A-transversion	Exon 1	Met61Lys	Autosomal dominant FTA	38
		Deletion entire MSX1 locus	No protein product from this chromosome	Wolf-Hirschhorn syndrome	40
	559C > T-transversion	Exon 2, homeobox domain	Gln187Stop	Autosomal dominant FTA	22
	62G-duplication	Exon 1	Frameshift after Gly (p.G22RfsX168)	Autosomal dominant FTA	29
PAX9	219insG	Exon 2, paired box domain	Frameshift at aa 73, termination of translation at aa 316	Autosomal dominant FTA	5
	340A > T-transversion	Exon 2 paired box domain	Lys114Stop	Autosomal dominant FTA	14
	793insC	Exon 4	Frameshift at aa 264, termination of translation at aa 315	Autosomal dominant FTA	15
		Deletion entire PAX9 locus	No protein product from this chromosome	Autosomal dominant FTA	17
	271A > G-missense	Exon 2, paired box domain	LYS91Glu	Autosomal dominant FTA	16
	62T > G-missense	Exon 2, paired box domain	Leu21Pro	Autosomal dominant FTA	16
	175ins288pb	Exon 2, paired box domain	Frameshift at aa 58, termination of translation at aa 177	Autosomal dominant FTA, 1 CLP person	16
	151G > A-transition	Exon 2, paired box domain	Gly51Ser	Non-FTA	18
	83G > C-missense	Exon 2, paired box domain	R28P	Autosomal dominant FTA	25
	76C > T-missense	Exon 2, paired box domain	Arg26Trp	Autosomal dominant FTA	19
	1A > G	Exon 1, start codon	No protein product from one gene copy	Autosomal dominant FTA	20
	109insG	Exon 2, paired box domain		Autosomal dominant FTA	21
	139C > T-missense	Exon 2, paired box domain		Autosomal dominant FTA	21
	619_621del A Tins24bp	Exon 2	Termination of translation at aa 210 Frameshift and termination of translation at aa 314	Autosomal dominant FTA	26
	259A > T-missense	Exon 2, paired box domain	Ile87Phe		27
AXIN2	C1966T-transversion	Exon 7	Arg656Stop	Autosomal dominant FTA CC	13
	1994–1995insG	Exon 7	Frameshift at aa 666, termination of translation at 706	Autosomal dominant FTA	13

Nucleotide numbers are relative to the translation initiation codon within the coding region.FTA, familial tooth agenesis; CP, cleft palate; CLP, cleft lip and palate; CC, colorectal cancer.

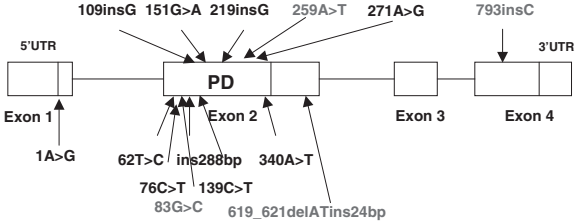

Fig. 2. Schematic view of human MSX1. HD = the region encoding the homeodomain; arrows indicate location of known mutations (28).

out of 55 tested probands. These variants seem to have an increased risk of selective tooth agenesis. More mutations of *AXIN2* should be studied to identify genotype/phenotype correlations and the specific pattern of tooth agenesis for this kind of mutations (29).

In vitro studies of the mutated MSX1 and PAX9 proteins show alterations in their protein structure, thereby often influencing the thermo-stability and/or

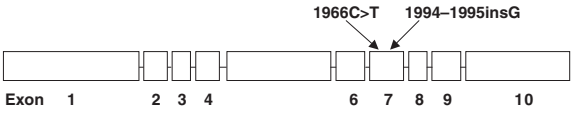

Fig. 3. Schematic view of human AXIN2. Arrows indicate the location of known mutations (13).

the three-dimensional folding. The normal functional activities of the mutant protein are disturbed; DNA-binding capacity and interactions with other transcriptional factors change. Sometimes the protein has no function at all (28). As the deletions of one copy of these genes cause the most severe phenotypes, it is probable that the known mutations are mostly loss-of-function mutations.

The allelic heterogeneity (different mutations within the same gene), as well as the locus heterogeneity (mutations in different genes) thus probably contribute to the large variation of phenotypes. But even within the same gene, the same allelic mutation and in the same family, different teeth can be missing through a dosage-sensitive mechanism of the mutated protein. Hypodontia obviously is a genetically very heterogeneous condition (28–34,42–46). Further functional *in vitro* characterization of mutated proteins are necessary to fully understand the different genetic mechanisms.

In our selective patient group no mutations could be discovered in the examined regions of the *PAX9*, *AXIN2* or *MSX1* genes. The one nucleotide change C > A that was found in one of our patients (Table 1) is actually far away from the PAX9 reading frame and rather seems to have an evolutionary conservation. However, it seems a rare allele, as it has so far (in our laboratory) only been detected in this patient. The same patient was also homozygous for the amino acid (Ala240Pro) change caused by a SNP in the third exon. Although there is a high chance that this and the two other SNPs found in our sample (Table 1) may not have clinical significance as such, they might be useful in efforts to pick specific haplotypes associated with increased risk for tooth agenesis. However, to test the importance of SNPs functional tests of much larger sample sizes are required.

In this perspective it is very likely that the cause for the severe oligodontia in our patients must be searched elsewhere. Alterations in the intronic regions of these genes or mutations in other genes encoding for growth and transcriptional factors like BMP4, FGF8, DLX, TBP, and others, which contribute significantly during odontogenesis, are important candidates. Defects in these genes could be an explanation for (our impression of) the discrepancy between the high incidence of tooth agenesis and the relatively low incidence of discovered and reported mutations in *PAX9*, *MSX1* and *AXIN2* genes in this and earlier examined risk groups. Also the less investigated mutations affecting mRNA processing might contribute to this discrepancy. Of course it is supposed that negative results of mutation screens are not often reported. Only a few reports can be found on the negative outcomes of tested patient groups for *MSX1*, *PAX9* or *AXIN2* mutations; Nieminen et al. (47) failed to identify linkage to *MSX1* in five unrelated families with hypodontia. Scarel et al. (48) could not discover any mutations in 20 patients with hypodontia. Frazier-Bowers et al. (49) also did not succeed to identify a *PAX9* nor *MSX1* mutation in 20 Vietnamese families. They also could not find any mutations in a high risk group of an earlier study (50). More consequent reporting about positive and negative results of mutation screening (the exact number of probands tested included) can give useful information about the real incidence of mutations in hypodontia patients. Therefore, patient screening for *PAX9*, *MSX1* and *AXIN2* mutations is still interesting and useful. Interest should however also go to other genes and developmental factors known from the mouse model.

In future studies more attention should also be paid on recently evidenced interactions of PAX9–MSX1 proteins as they maybe could explain the negative mutation results as in the present study (28).

Conclusion

Oligodontia is a heterogeneous condition, which is in the eight families of this study not caused by *PAX9*, *AXIN2* or *MSX1* mutations. *PAX9*, *MSX1*, *AXIN2* and other genes regulating odontogenesis need further *in vivo* and *in vitro* examination to explain the phenotypic heterogeneity and to increase our understanding of the odontogenic processes. Positive as well as negative research results, the exact number of probands tested included, should be reported in this respect.

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