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Mutational analysis of *MSX1* and *PAX9* genes in Portuguese families with maxillary lateral incisor agenesis

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SUMMARY The observation that certain patterns of tooth agenesis occur more frequently in individuals of the same family may suggest the existence of predisposing genetic factors. The aim of this study was to search for mutations in the *PAX9* and *MSX1* genes and to investigate their potential association with the maxillary lateral incisor agenesis (MLIA) phenotype in 12 Portuguese families, a total of 52 individuals, 12 probands and 40 relatives (eight of which had MLIA). Twenty-three of the subjects were male and 29 female with an age range of 10–75 years. The control group comprised random DNA samples of 91 Portuguese individuals.

Nucleotide alterations were not detected in the coding regions of the *MSX1* gene, analysed by single-strand conformation polymorphism and sequencing; in the *PAX9* gene, a polymorphism was found that led to transition of G718 to C, implying a change of alanine 240 for proline. However, the differences in the frequencies of the *PAX9* gene polymorphism between the probands (67 per cent) and the control population (56 per cent carrying the c allele) were not statistically significant as determined by chi-square test, and the polymorphism did not clearly segregate with the trait in the families. Aggregating the available data, there does not seem to exist a clear association between the alanine 240 for proline variant in the *PAX9* gene and the MLIA phenotype. Further studies are required to clarify the basic genetics of MLIA.

Introduction

Congenital absence of a maxillary lateral incisor and of mandibular second premolars are the two most frequent forms of hypodontia, their frequency varying with the population studied (Grahnén, 1956; Horowitz, 1966; Alvesalo and Portin, 1969; Muller *et al.*, 1970; Thilander and Myrberg, 1973; Magnusson, 1977; Rolling, 1980; Aasheim and Øgaard, 1993; Johannsdottir *et al.*, 1997; Tavajohi-Kermani *et al.*, 2002; Polder *et al.*, 2004). Values ranging between 0.8 and 4.25 per cent have been found for the prevalence of permanent maxillary lateral incisor agenesis (MLIA). In the Portuguese population, prevalence of MLIA was estimated at 1.3 per cent (Pinho *et al.*, 2005). Svinhufvud *et al.* (1988) and Arte *et al.* (2001) have proposed that dental absence, microdontia, or the conical form of one or more maxillary lateral incisor are different expressions of the same trait.

In spite of recent progress, the aetiopathogenesis of hypodontia remains largely unknown (Vastardis, 2000; Kapadia *et al.*, 2007). There is evidence that congenital tooth absence can be the result of environmental or hereditary causes, or of their interaction (Schalk-van der Weide and Bosman, 1996; Swinnen *et al.*, 2008). However, as the development of the human dentition in terms of structure and organization is under genetic control and involves several factors (Lyngstadaas *et al.*, 1996; Pirinen *et al.*, 1996; Thesleff, 1996; Vastardis *et al.*, 1996; Stockton *et al.*, 2000; Vastardis, 2000; Nieminen *et al.*, 2001; Das *et al.*, 2002; Lidral and Reising, 2002; Klein *et al.*, 2005; Gerits *et al.*, 2006; Mostowska *et al.*, 2006; Kapadia *et al.*, 2007; Tallon-Walton *et al.*, 2007; Zhao *et al.*, 2007), it is logical to assume that mutations in some genes encoding these factors may affect the normal development of teeth and, eventually, may cause their absence.

Inactivation of *MSX1* and *PAX9* genes in mice showed that these genes are relevant to dental and craniofacial development (Satokata and Maas, 1994; Neubuser *et al.*, 1997). Some mutations associated with tooth agenesis have been identified in humans in the *MSX1* (Vastardis *et al.*, 1996; Lidral *et al.*, 1998; Lidral and Reising, 2002; Mostowska *et al.*, 2006) and *PAX9* (Schuffenhauer *et al.*, 1999; Stockton *et al.*, 2000; Nieminen *et al.*, 2001; Das *et al.*, 2002; Pereira *et al.*, 2006; Hansen *et al.*, 2007; Tallon-Walton *et al.*, 2007; Zhao *et al.*, 2007; Guala *et al.*, 2008) genes. Vastardis *et al.* (1996) identified one missense mutation in *MSX1* (Arg31Pro), resulting in the substitution of arginine by proline in position 31 of the peptide chain, in all affected members of one family, with congenital absence of the second premolars and third molars as a predominant characteristic. Some affected individuals also presented

congenital absence of the first premolars, first mandibular molars, one or both maxillary lateral incisors, or one mandibular central incisor.

Van den Boogaard *et al.* (2000) found a nonsense mutation (Ser104Stop) in the *MSX1* gene in all affected individuals of a Dutch family with several combinations of orofacial clefting and dental agenesis (predominantly of the second premolars and molars). This phenotype is similar to that which occurs as the result of *MSX1* gene mutation in mice (Satokata and Maas, 1994).

Nevertheless, Nieminen *et al.* (1995), in a study of families with slightly different clinical features (incisor and premolar hypodontia), excluded the *MSX1* and *MSX2* genes as potentially implicated in hypodontia, as well as epidermal growth factor (EGF), *EGFR* and *EGF-3* (Arte *et al.*, 1996). A study performed in individuals of Brazilian origin with hypodontia failed to find mutations in *MSX1* (Scarel *et al.*, 2000). Lidral and Reising (2002) did not find mutations in *MSX1* in subjects with simple incisor and premolar agenesis nor in those with orofacial clefts, but they did detect them in families with multiple agenesis, indicating a different aetiology for these cases. Mostowska *et al.* (2006) described a novel mutation of *MSX1* that might be responsible for oligodontia (lack of 14 permanent teeth in a proband), although with incomplete penetrance.

Peters *et al.* (1998), in animal studies of gene inactivation, showed that the expression of transcription factors dependent on PAX9 regulation was essential to the normal development of structures derived from the pharyngeal pouch, such as the craniofacial skeleton, teeth, and fingers. Affected mice died in the prenatal period, presumably due to serious craniofacial malformations, including palatal fissures. Peters *et al.* (1998) also showed that the *PAX9* gene is fundamental for the mesenchymal expression of transcription factors: bone morphogenetic protein 4 (Bmp4), MSX1, and lymphoid enhancer-binding factor 1 (Lef1), suggesting a role for this gene in the establishment of the inductive capacity of the mesenchyme in tooth formation.

In humans, mutations in *PAX9* were identified in individuals with oligodontia, affecting mostly the molars, suggesting the importance of the expression of that gene for the formation of the dentition (Stockton *et al.*, 2000; Nieminen *et al.*, 2001; Pereira *et al.*, 2006; Hansen *et al.*, 2007; Tallon-Walton *et al.*, 2007). Lammi *et al.* (2004), recently, reported that a nonsense mutation in *AXIN2*, an essential component of the WNT/β-catenin pathway, caused familial oligodontia with a severe phenotype. In addition to oligodontia, those authors also found that a mutation in *AXIN2* predispose the individual to colorectal cancer. However, considering the discrepancy between the high prevalence rate of tooth agenesis and the relatively small number of reported causative mutations in the *PAX9*, *MSX1*, and *AXIN2* genes, the genetic contribution to hypodontia/oligodontia seems quite heterogeneous (Gerits *et al.*, 2006). Environmental and epigenetic factors as well as genes regulating odontogenesis require further *in vivo* and *in vitro* investigation in order to explain the phenotypic heterogeneity and to increase understanding of the odontogenic processes (Swinnen *et al.*, 2008).

A study of familiar aggregation showing that the risk of a relative of an individual with maxillary lateral incisor agenesis (MLIA) having the same type of agenesis was 13–15 times higher when compared with a relative of an individual without agenesis (Pinho, 2004) has reinforced the hypothesis that genetic changes may lead to congenital absence of teeth. Thus, the current study aimed to evaluate a possible association between mutations in *MSX1*, *PAX9*, and the MLIA phenotype in patients of Portuguese origin.

Subjects and methods

All participants gave their written informed consent to participation in this study, which was approved by the Ethics Committee of the Faculty of Dental Medicine of the University of Porto.

Patients and controls

A previous epidemiological study involving analysis of 16 771 patients between 1993 and 2000 at a university clinic led to the identification of 219 subjects with permanent MLIA, corresponding to a prevalence of 1.3 per cent in this population (Pinho *et al.*, 2005).

Comprehensive clinical information, family history, and radiographs were obtained for 52 of the above-mentioned individuals, 12 probands, and 40 relatives (eight of which had MLIA; families 1–12); 23 (44.2 per cent) of the subjects were male and 29 (55.8 per cent) female, and their age ranged from 10–75 years (mean 33.48 ± 17.37; Figure 1, available as supplementary data on *European Journal of Orthodontics* online). Care was taken during clinical observation to exclude known genetic syndromes associated with tooth absence, and records were made of all skin, hand, eye, lip, and palatal anomalies, as well as hearing problems. Random DNA samples of 91 Portuguese individuals (obtained from anonymous Guthrie cards, from the national newborn screening programme) constituted the control population.

Polymerase chain reaction amplification

Genomic DNA was extracted from peripheral blood using the Gentra PureGene Blood kit (Gentra Systems, Minneapolis, Minnesota, USA) and from the buccal smears or filter paper blots using Chelex at a final concentration of 0.8 per cent.

Four sets of primers were synthesized, spanning the coding region of *MSX1* (Exons 1 and 2) and of the *PAX9* gene (Exons 2, 3, and 4) and standard polymerase chain reaction (PCR) amplification of genomic DNA was used. PCR was performed in a total volume of 25 μl containing 40 ng of DNA, 1 μM of each primer, 25 mM $MgCl_2$, 200 μM deoxyribonucleotides, and 0.01 U of DNA Taq polymerase (Fermentas GmbH, St Leon-Rot, Germany), in

100 mM Tris–HCl, pH 8.8, 500 mM KCl buffer. Samples were initially heated to 95°C for 5 minutes. Each cycle comprised denaturation at 94°C for 1 minute, primer annealing at optimal annealing temperature for 30 seconds, and extension at 72°C for 1.5 minutes. The samples were subjected to 35 cycles of amplification, followed by a final extension at 72°C for 7 minutes. The PCR products were analysed in 2 per cent agarose gel.

Mutation scanning by single-strand conformation polymorphism analysis

A non-radioactive single-strand conformation polymorphism (SSCP) method was used to scan for sequence variants in the coding region of the *MSX1* gene and the second exon of the *PAX9* gene.

Amplified DNA from these segments was subjected to electrophoretic migration on 11 per cent polyacrylamide gel with glycerol, under non-denaturing conditions in the Multiphor® apparatus (GE Healthcare Life Sciences, Amersham, Buckinghamshire, UK). Different temperatures were used for electrophoretic separation for each gene segment: 15°C for *PAX9*, 4°C for Exon 1, and 15°C for Exon 2 of the *MSX1* gene. The gel was silver stained, using standard methods. If any differences were detected, direct sequencing was carried out as described below.

DNA sequencing

PCR products were sequenced using the PCR Pre-Sequencing Kit (GE Healthcare Life Sciences), according to the manufacturer's instructions. Complete sequencing of the coding regions of *MSX1* (Exons 1 and 2) and *PAX9* gene (Exons 2, 3, and 4) was performed through automated cycle sequencing (ABI Prism® Big Dye Terminator Cycle Sequencing Ready Reaction kit; Applied Biosystems, Foster City, California, USA), according to the manufacturer's instructions, using the primers listed in Table 1. Capillary electrophoresis was performed in an ABI 310 (Applied Biosystems).

Allele-specific PCR

Allele-specific PCR was used to confirm the presence of a mutation in the probands and family members and to study the frequency of DNA variants in the control population. The PCR started with denaturation at 95°C for 5 minutes, followed by 32 cycles of denaturation at 94°C for 1 minute, annealing at 62°C for 30 seconds, and extension at 72°C for 1 minute. A final extension at 72°C for 5 minutes was performed. The reaction occurred in a total volume of 12.5 µl, containing 100 mM Tris–HCl, pH 8.8, 500 mM KCl buffer, 1.5 mM $MgCl_2$, 0.6 mM deoxyribonucleotides, 0.03 per cent formaldehyde, and 0.01 U Taq polymerase (Gibco-Invitrogen, Carlsbad, California, USA). Subjects of known genotype were used in each run to validate the specificity of PCR. The amplification products were detected in 2 per cent agarose gel. Figure 2 shows an example of the results obtained with this method.

Table 1 Sequence of primers used for amplification of the different exons and conditions in polymerase chain reaction. F, forward; R, reverse; ex, exon; numeration, nucleotide position in the sequence; Ta, annealing temperature (matching temperature).

Gene	Primers	Sequence	Size (base pairs)
MSX1	4Fex1	AGTCGCCAGAGGAAAGTTTC	650
	650Rex1	CCTTGGCCCCCGGCTTCCTGTGGTC	
	643Fex1	CCAAGGAGAGCGCCCTGGCGCCCT	277
	920Rex1	CTCCCTCTGCGCCTGGGTTCTGGCT	
	645Fex2	GCAAACACAAGACGAACCGTAAGCC	200
	840Rex2	CTTGTAGTCTCTTTGCCTTGGCGCG	
	570Fex2	CAATGCTTCTCTCTTAACCCTTGCTT	520
	1090Rex2	CTGGCCCCACAGGTGGGCGGACCTG	
PAX9	−58F1ex2	AGGCAGCTGTCCCAAGCAGCG	410
	357R1ex2	GGAGGGCACATTGTACTTGTCGC	
	109F2ex2	ATCCGACCGTGTGACATCAGCC	525
	+10R2ex2	GAGCCCCTACCTTGGTCGGTG	
	−197Fex3	GGGAGTAAAACTTCACCAGGC	370
	+28Rex3	CCACCTGGCCTGACCCTC	
	−121Fex4	GGAGAGTAGAGTCAGAGCATTGCTG	590
	+74Rex4	GAGACCTGGGAATTGGGGGA	

Extracted and adapted from Vastardis *et al.* (1996) and Nieminen *et al.* (2001).
(+) indicates a sequence of DNA that is in front of the codon where transition begins and (−) the sequence of DNA that is behind the codon.
The annealing temperature of the primers used was 65°C for all segments to be amplified.

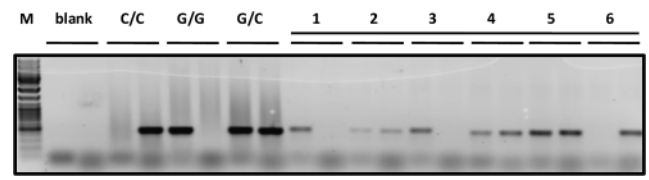

Figure 2 Agarose gel electrophoresis showing the allele-specific polymerase chain reaction (PCR) for the study of substitution in the 718 nucleotide position (G>C): Lanes 1 and 3: G/G homozygous normal genotype; Lanes 2, 4, and 5: G/C heterozygous genotype; and Lane 6: C/C homozygous mutant genotype. To the left are the negative blank and positive controls of the allele-specific PCR.

Statistical analysis

Statistical analysis of the data was performed using the Statistical Package for Social Sciences version 16 (SPSS Inc., Chicago, Illinois, USA). A chi-square test was used to compare the frequency of mutations/polymorphisms between the probands and controls and between affected and non-affected relatives (excluding spouses). A *P*-value less than 0.05 was considered to be statistically significant.

Results

In the proband of each family, Exons 2, 3, and 4 of the *PAX9* gene and Exons 1 and 2 of the *MSX1* gene were sequenced, with the aim of identifying mutations in these genes. In

these 12 probands, no nucleotide changes were detected in the regions of the *MSX1* gene analysed by SSCP or sequencing. Thus, for this gene, no further mutation analysis was performed in the relatives.

Two sequence variants were identified in Exon 3 of *PAX9*: a silent change in nucleotide 717C>T=rs12881240 in one of the probands, and a variant in nucleotide 718G>C=rs4904210 (Figure 3) leading to the Ala240Pro substitution, in eight probands (66.7 per cent of probands), four homozygous, and four heterozygous. In the control population of Portuguese ancestry, the frequency of the Ala240Pro variant was 56 per cent. The observed differences in the frequencies of this polymorphism between the probands and controls were not statistically significant (chi-square test $P = 0.1$).

Analysis of these specific variants in *PAX9* was also performed by allele-specific PCR (Figure 2) in the remaining 40 family members, eight of which were affected with MLIA. The Ala240Pro variant was found in 28 (70 per cent of the relatives, nine homozygous and 19 heterozygous) (Figure 1, Table 2, available as supplementary data in *European Journal of Orthodontics* online).

Analysis of the distribution of the Ala240Pro polymorphism genotypes in *PAX9* in the eight families with MLIA revealed no difference ($P = 0.12$) in the frequency of homozygous and heterozygous carriers of this polymorphism compared with that seen in the control population (Table 3). Additionally, analysis of the frequency of the Ala240Pro polymorphism and corresponding genotypes in affected and non-affected cases (excluding individuals who married into the basic gene pool; $n = 34$, Table 3) was performed of the 12 families. No significant difference was found in the distribution of the Ala240Pro polymorphism between the affected and non-affected groups, either when considering as affected with agenesis only, or extending the concept of 'affected' to include those with agenesis and/or microdontia. ($P > 0.05$).

Discussion

In this study, no mutations in the *MSX1* gene were found in individuals with MLIA, while a polymorphism in the *PAX9* gene was detected, the frequency of which was not, however, statistically different from that in the control population.

This result reinforces the view that the *MSX1* gene might be fundamentally implicated in odontogenesis of posterior teeth (Nieminen *et al.*, 1995; Vastardis *et al.*, 1996; Scarel *et al.*, 2000; Vastardis, 2000; Nieminen *et al.*, 2001;

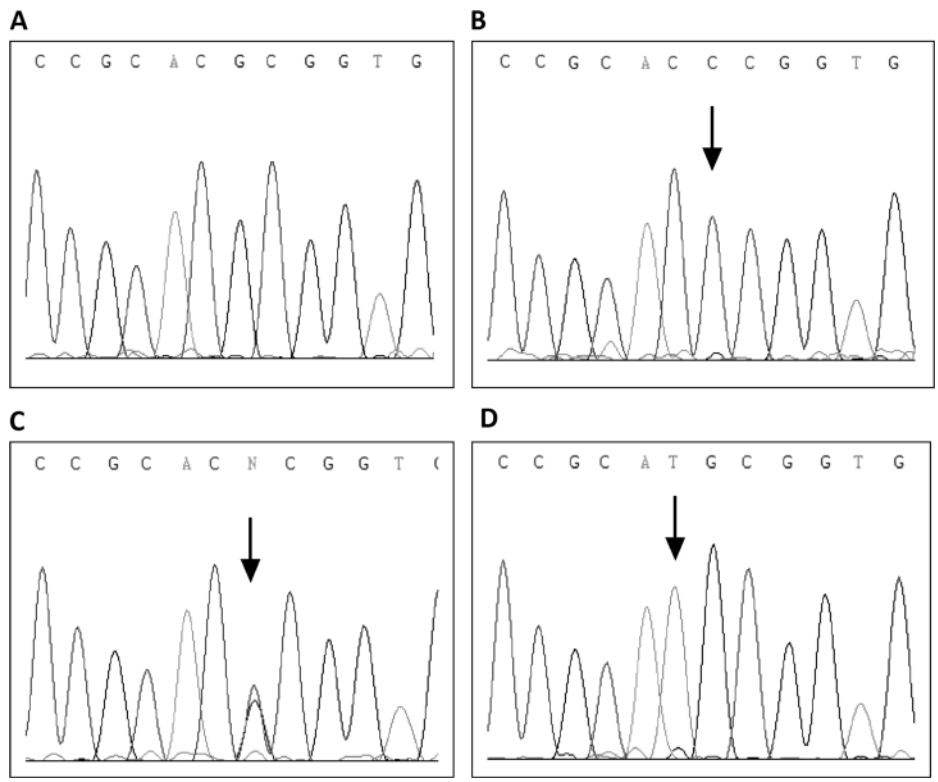

Figure 3 Chromatogram showing the sequencing analysis of Exon 3 of *PAX9*, from four probands. (A) wild-type sequence, (B) homozygosity, (C) heterozygosity for the G>C substitution at nt 718, and (D) homozygosity for the silent polymorphism at nt717(C→T).

Table 3 Frequencies of the different genotypes (G/G, C/C, and G/C) observed in the *PAX9* gene polymorphism G718C of controls, subjects with maxillary lateral incisor agenesis and their relatives.

Studied population	Total (*n*)	*PAX9* G718C substitution		
		No	Yes	
		Homozygote normal genotype (G/G)	Homozygous (C/C)	Heterozygous (G/C)
Probands (individuals with incisor agenesis)	12	4 (33.33%)	4 (33.33%)	4 (33.33%)
Relatives of probands*	34	9 (26.47%)	8 (23.53%)	17 (50%)
With agenesis	8	4 (50%)	2 (25%)	2 (25%)
Without agenesis	26	5 (19.23%)	6 (23.08%)	15 (57.69%)
Controls (individuals without incisor agenesis)	91	40 (43.96%)	14 (15.38%)	37 (40.66%)

*Spouses and individuals of unknown phenotype were excluded from the analysis.

De Muynck *et al.*, 2004; Mostowska *et al.*, 2006). Illustrating this concept is the description by Mostowska *et al.* (2006) of a novel mutation of *MSX1* with incomplete penetrance, potentially responsible for oligodontia in a proband with absence of 14 permanent teeth but with the presence of the maxillary incisors. Those authors supported the view that oligodontia of the human dentition might be an oligogenetic trait caused by simultaneous mutation of different genes.

Mutations in the *PAX9* gene have been associated with agenesis of posterior teeth (Stockton *et al.*, 2000; Nieminen *et al.*, 2001; Pereira *et al.*, 2006; Hansen *et al.*, 2007; Tallon-Walton *et al.*, 2007). In humans, unlike mice, a heterozygous mutation in either *PAX9* or *MSX1* was reported to be sufficient to cause agenesis of molars or premolars, respectively (De Muynck *et al.*, 2004; Kapadia *et al.*, 2007). In the present study, the location of the agenesis studied was at an anterior level, and a sequence variant in the *PAX9* gene [nt718 (G>C)] was identified. This nucleotide substitution has previously been described (Nieminen *et al.*, 2001), leading to the introduction of a proline instead of alanine in the peptidic chain. The alanine residue at position 240 is conserved during evolution and is present in several species (namely *Gallus gallus, Mus musculus, Danio rerio, Takifugu rubripes*), being maintained in rodents and in mammals (Pereira *et al.*, 2006). Its substitution by proline could be a reason for the structural change in the PAX9 protein with a corresponding functional effect. It has been suggested that in spite of a globally strong purifying selection acting upon *PAX9*, the Ala240Pro polymorphism may confer an evolutionary advantage by causing congenital absence of third molars in humans (Pereira *et al.*, 2006). In agreement with this hypothesis, this variant was common but was not significantly more frequent in individuals with MLIA than in the control population in the present study. Homozygosity for the variant was not significantly more frequent in the MLIA probands than in the controls. Segregation analysis and the correlation between genotype–phenotype in the MLIA families also did not reveal an association of the nt718 (G>C) or of the Ala240Pro variants of *PAX9* with hypodontia. Family members without agenesis and some healthy partners were also heterozygous for the Ala240Pro polymorphism, reflecting its frequency in a large proportion of the control population.

In the controls, in the present study, the frequency of the Ala240Pro variation was 56 per cent, close to that recently reported of 69.8 per cent for a southeast Chinese control population (Pan *et al.*, 2008). In the latter population, the same *PAX9* polymorphism alone had a non-significant effect on the overall risk of tooth agenesis because no significant difference was found in the haplotype distribution between the controls and subjects with tooth agenesis or mandibular incisor agenesis. A specific haplotype of this gene was, however, reported to have a protective effect, being associated with a decreased risk of tooth agenesis (Pan *et al.*, 2008).

Although previous research of familial aggregation (Pinho, 2004) indicated an increased risk of MLIA in relatives of affected probands, it was not established in this study that *MSX1* and *PAX9* genes have a relevant aetiopathogenic implication for this developmental defect, at least in the Portuguese population. This implies that several other genes are possibly implicated in the pathogenesis of dental agenesis.

Conclusions

Sequence variants in the *MSX1* and *PAX9* genes, including the G718C polymorphism, were not the cause of hypodontia in the studied patients. The phenotype of affected probands might result from mutations of other developmentally relevant candidate genes, yet to be identified.

Supplementary material

Supplementary material is available at *European Journal of Orthodontics* online.

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