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Original Research

Effects of PAX9 and MSX1 gene variants to hypodontia, tooth size and the type of congenitally missing teeth

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Abstract: Tooth agenesis, affecting up to 20% of human population, is one of the most common congenital disorder. The most frequent form of tooth agenesis is known as hypodontia, which is characterized by the absence of one to five permanent teeth excluding third molars. It was considered that hypodontia is especially related with gene mutations which play role in tooth formation. Additionally mutations in *PAX9* and/or *MSX1* have been identified as the defects responsible for missing permanent molars and second premolars. In some studies it was also found that *PAX9* and *MSX1* gene mutations may change tooth size. Therefore in this study all of these factors were investigated. Thirty one patients and 30 controls were enrolled to the study. Information about tooth sizes and type of congenitally missing teeth were collected. *MSX1* and *PAX9* gene mutations were investigated by direct sequencing. Results were evaluated statistically. As a result, 22 variations were detected in *PAX9* in which 18 of them are novel. In addition, 7 variations were found in *MSX1* in which 5 of them are novel and one of them lead to amino acid change. Statistically significant relations were found between detected variations and tooth sizes. Any relation between mutations and type of congenitally missing teeth were not detected. In conclusion, especially new mutations which may cause hypodontia, effect tooth size and type of congenitally missing teeth, should be investigated with other researchers for clarifying the mechanism.

Key words: Hypodontia, *PAX9*, *MSX1*, tooth size.

Introduction

Agenesis of permanent teeth constitutes one of the most common developmental abnormalities in humans characterized by the developmental absence of one or more teeth (1). The most common permanent teeth missing are the third molars (20%), second premolars (3.4%), and maxillary lateral incisors (2.2%) (2). Tooth agenesis is divided into three categories. Hypodontia is defined as the absence of one to five permanent teeth, excluding third molars, whereas the absence of more than six teeth is referred as oligodontia. The most extreme case is anodontia, denoting absence of all teeth (3). Among these, hypodontia is one of the most frequent alterations of the human dentition (2). Although hypodontia does not represent a serious public health problem, it may cause masticatory and speech dysfunctions, and also esthetic problems.

Tooth agenesis is classified as sporadic or familial form, inherited as autosomal dominant (4), autosomal recessive (5), or an X-linked (6). It may occur as a part of a genetic syndrome, or as a nonsyndromic disorder (7). It has been demonstrated that nonsyndromic form of familial and sporadic tooth agenesis is associated with mutations in Muscle Segment Homeobox 1 (*MSX1*) and Paired Box 9 (*PAX9*) (8-17). MSX1 and PAX9 are both transcription factors expressed in the dental mesenchyme at the stage of initiation of tooth development, in both dental follicle and dental papilla (18, 19).

The *PAX9* gene is localized in chromosome 14 (14q12–q13), and encodes a protein containing 341 amino acids (20, 21). It is a member of a gene family that play a key role during embryogenesis (1). It is directly involved in the craniofacial development, particularly in the formation of the teeth and palate. The majority of mutations identified are located at the paired domain coding region, which corresponds to the DNA-binding site of PAX9 factor. Generally, these mutations affect major signaling pathways mediated by PAX9 and other transcription factors during odontogenesis. Accordingly, abnormalities in the odontogenesis could occur including the arrest of tooth bud (22). Mutations in the paired box gene *PAX9* have been identified as the defects responsible for missing permanent molars (7).

The human *MSX1* gene is located at chromosome 4p16.1 (23). It is expressed in 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, also interacts with other components during the signalling pathways of odontogenesis, like the TATA-binding protein (TBP) or DLX-family (24). Homozygous *MSX1*-deficient mice exhibit craniofacial deformities like deficiencies in mandibular and maxillary alveolar processes, secondary cleft palate, and disturbed tooth development during transition from bud to

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cap stage (25-27). Mutations in the *MSX1* homeobox gene were shown to cause human autosomal dominant agenesis, primarily in third molars and second premolars (8).

Studies have shown that a key function of the signalling pathway involving MSX1 and PAX9 is the maintenance and regulation of mesenchymal Bmp4, which is critical for the progress of tooth morphogenesis from the bud stage to the cap stage (28, 29). PAX9 interacts with MSX1 at both gene and protein levels, and this interaction enhances the ability of PAX9 to transactivate BMP4 and MSX1 expression (28, 30).

Most of the mutations in hypodontia patients are located in the *PAX9* paired box domain (a segment of 381 bp inside exon 2) and in the *MSX1* homeodomain (segment of 180 bp inside exon 2) regions that are responsible for coding the DNA binding regions (31). Thus in this study whole exon 2 of *MSX1* and *PAX9*, and partially non-coding regions of both genes were investigated. Additionally, there is a wide variation in tooth size and the location of congenitally missing teeth in patients. Therefore at the end of the study the effects of *MSX1* and *PAX9* gene mutations to hypodontia, tooth size and the type of congenitally missing teeth were evaluated.

Materials and Methods

Subjects

Thirty one unrelated individuals with selective tooth agenesis who showed no signs of other congenital abnormalities or systemic diseases were recruited from the Department of Orthodontics, Faculty of Dentistry, Yeditepe University. Medical situations, birth defects, and family histories were gathered to identify possible associated anomalies and to differentiate the non-syndromic from the syndromic agenesis. The inclusion criterion was congenital agenesis of at least 1 permanent tooth, not including third molars, as verified by dental history and panoramic X-ray analysis. No other dental anomalies were observed in the subjects. Thirty individuals with normal number and shape of teeth were recruited as controls.

Tooth sizes were measured from plaster models available from all cases and controls. The maximal mesiodistal and buccolingual widths of each tooth were measured with a high-precision digital caliper (Digital Calipers; Masel, Henry Schein Orthodontics) according to the method of Alvesalo (32). The present study complies with the Declaration of Helsinki and was approved by the Institutional Ethics Committee of Yeditepe University, Istanbul, Turkey. All individuals gave written informed consent prior to study inclusion.

Molecular analysis

Total genomic DNA was extracted from peripheral blood leukocytes collected from each subject, into EDTA-tubes, using the High Pure PCR Template Preparation Kit (Roche, Basle, Switzerland), according to manufacturer's instructions. Nucleotide numbering of *PAX9* as well as MSX1 begins from the start codon of the each gene.

PCR Amplification

Partially intron 1, intron 2 and 3´UTR regions of *PAX9* and *MSX1* as well as whole exon 2 of *PAX9* and *MSX1* were amplified by polymerase chain reaction (PCR) using 50–100 ng of total DNA. Table 1 and 2 lists the sequences of primers which were used for amplifying *PAX9* and *MSX1* related regions respectively by PCR. PCR amplifications were performed in a total volume of 50 µl containing 50–100 ng DNA template in 10 mM Tris–HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 100 mM each of dNTPs, 1.0U Taq DNA polymerase, and 1.0 mM of each primer. The conditions of PCR amplification for *PAX9* are as follows: a denaturation step at 95 °C for 3 min followed by 35 cycles at 95 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min, a final extension at 72 °C for 5 min, and a stop at 4 °C. The conditions of PCR amplification for *MSX1* are as follows: a denaturation step at 95 °C for 3 min followed by 35 cycles at 95 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min, a final extension at 72 °C for 5 min, and a stop at 4 °C. All PCR products were fractionated by electrophoresis on a 2% agarose gel. The primers used for the amplification of *PAX9* and *MSX1* are shown in Table 1.

Purification of PCR Products and Direct Sequencing of PAX9 and MSX1 regions

All PCR products were purified by using the High Pure PCR Product Purification Kit (Roche), according to the manufacturer's instructions before direct sequencing. Then, purified PCR products were sequenced by using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham, Buckinghamshire, UK) in ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing protocol was also carried out with reverse primers for confirmation.

Statistical analysis

SPSS 23.0 were used for statistical analysis. Descriptive analyses were presented using means and standard deviations for contionus data and frequecies and percentages for categorical data. The variables inverstigated using Kolmogorov Smirnov test to determine whether or not they are normally distributed. Mann-Whitney U test was used to compare the patient and control groups. The Chi-Square and Fisher's exact test, where appo-

Table 1. PCR primers used for the amplification of *PAX9* and *MSX1*.

Amplificated fragments of *PAX9* and *MSX1*	PCR Primers
PAX9 (865 bp.)	5'-primer: 5'- TGTTCAGGGACCATATGGTTT -3'
	3'-primer: 5'- TCCCTGAGGCTGCAGATACT -3'
MSX1 (858 bp.)	5'-primer: 5'- TTACTACTTCTTGGGCTGATCAT -3'
	3'-primer: 5'- AGGGAAAAGCTATGCAGGAGA -3'

Table 2: Locations of congenitally missing permanent teeth of patients.

		Congenitally missing permanent teeth											
		Left						Right					
Patient number	Dental arch	6	5	4	3	2	1	1	2	3	4	5	6
1	Maxillary					x							
	Mandibular												
2	Maxillary					x							
	Mandibular												
3	Maxillary												
	Mandibular					x			x				
4	Maxillary					x			x				
	Mandibular												
5	Maxillary					x			x				
	Mandibular												
6	Maxillary												
	Mandibular			x							x		
7	Maxillary					x			x				
	Mandibular												
8	Maxillary			x									
	Mandibular								x				
9	Maxillary					x			x				
	Mandibular												
10	Maxillary					x			x				
	Mandibular												
11	Maxillary					x			x				
	Mandibular												
12	Maxillary					x			x				
	Mandibular												
13	Maxillary											x	
	Mandibular		x									x	
14	Maxillary												
	Mandibular						x						
15	Maxillary		x	x								x	
	Mandibular												
16	Maxillary					x							
	Mandibular			x							x		
17	Maxillary		x			x			x			x	
	Mandibular												
18	Maxillary					x			x				
	Mandibular												
19	Maxillary		x						x			x	
	Mandibular												
20	Maxillary					x			x				
	Mandibular			x							x		
21	Maxillary					x			x				
	Mandibular		x									x	
22	Maxillary					x			x				
	Mandibular											x	
23	Maxillary											x	
	Mandibular												
24	Maxillary		x			x			x			x	
	Mandibular											x	
25	Maxillary												
	Mandibular					x			x				
26	Maxillary					x							
	Mandibular			x							x		
27	Maxillary					x			x				
	Mandibular												
28	Maxillary					x			x				
	Mandibular		x									x	
29	Maxillary		x	x		x					x	x	
	Mandibular		x								x		
30	Maxillary												
	Mandibular		x									x	
31	Maxillary												
	Mandibular	x											x

priate, were used to compare genotypes. Since the variables are not normally distributed, Kruskal-Wallis test were conducted to compare these parameters among groups. Mann-whitney U test was performed to test the significance of pairwise differences using Bonferroni correction adjust for multiple comparasions p-values less than 0.05 ($p < 0.05$) were considered to be statistically significant.

Results

Clinical examinations

Locations of congenitally missing permanent teeth of patients are shown in Table 2. It was found that mostly lateral incisors, first and second premolars are missing.

When tooth sizes were compared between groups, some statistically significant results were found. Details are shown in Table 3.

Direct sequencing results

Twenty two variations were detected in *PAX9* in which 18 of them are novel. In addition, 7 variations were found in *MSX1* in which 5 of them are novel and one of them lead amino acid change. Representative DNA sequencing chromatograms of *PAX9* and *MSX1* are shown in Figure 1 and Figure 2, respectively.

Comparision of variations between groups

Table 4 summarize all of the information about mutations/polymorphisms in *PAX9* and *MSX1* which were found with direct sequencing. When variations were compared between groups 665 C-ins., 687 C-ins., 754 A>G, 1451 G-ins., 1475 G>A, 1509 A-ins. in *PAX9*; 3232 C>T and 3502 A>G in *MSX1* were found statisti-

Table 3. Comparision of tooth sizes between groups.

Tooth location		Tooth sizes of groups (mm) (Mean ± SD)		p values
		Control group (n=30)	Patient group (n=31)	
Maxillary				
Left	6	10,94 ± 0,44	10,94 ± 0,67	p=0.988
	5	6,62 ± 0,54	7,19 ± 0,62	**p=0.001***
	4	6,49 ± 0,32	7,21 ± 0,62	**p<0.001***
	3	8,33 ± 0,44	7,79 ± 0,58	**p<0.001***
	2	6,7 ± 0,51	6,48 ± 0,64	p=0.315
	1	8,58 ± 0,57	8,10 ± 0,93	**p=0.011***
Right	1	8,63 ± 0,58	8,18 ± 0,68	**p=0.001***
	2	6,72 ± 0,50	6,49 ± 0,54	p=0.21
	3	8,32 ± 0,42	7,73 ± 0,52	**p<0.001***
	4	6,48 ± 0,34	7,12 ± 0,57	**p<0.001***
	5	6,6 ± 0,57	7,19 ± 0,66	**p=0.002***
	6	10,9 ± 0,46	10,77 ± 0,65	p=0,292
Mandibular				
Left	6	11,08 ± 0,44	10,87 ± 0,67	p=0,225
	5	6,58 ± 0,62	7,23 ± 0,66	**p=0.001***
	4	6,46 ± 0,36	6,91 ± 0,69	**p=0.009***
	3	5,88 ± 0,56	6,42 ± 0,61	**p<0.001***
	2	5,47 ± 0,55	5,76 ± 0,53	**p=0.039***
	1	5,28 ± 0,35	5,35 ± 0,40	p=0.471
Right	1	5,26 ± 0,36	5,35 ± 0,35	p=0.357
	2	5,44 ± 0,49	5,78 ± 0,52	**p=0.013***
	3	5,88 ± 0,55	6,47 ± 0,64	**p<0.001***
	4	6,51 ± 0,43	7,04 ± 0,67	**p=0,002***
	5	6,64 ± 0,68	7,15± 0,64	**p=0.009***
	6	11,10 ± 0,45	10,84 ± 0,61	p=0.124

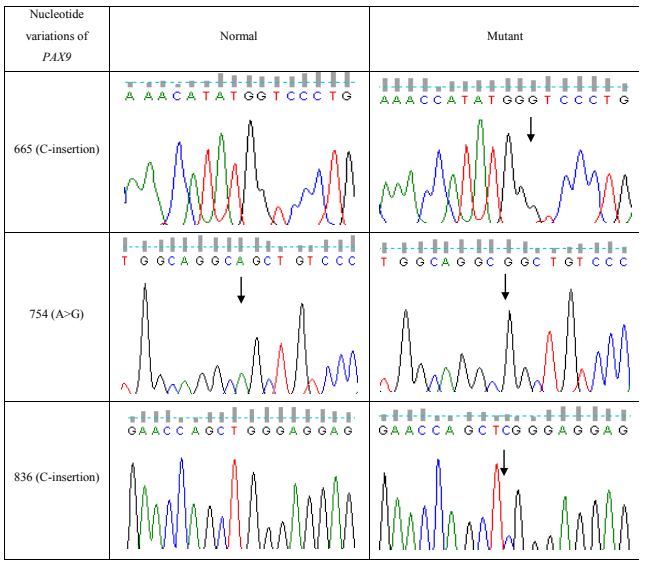

Figure 1. Representative DNA sequencing chromatograms showing mutations/polymorphisms of *PAX9*.

cally significant in patients.

Association between variations and the type of congenitally missing teeth

However mostly lateral incisors, first and second premolars are missing, any relation between variations and the type of congenitally missing teeth were not found.

Relation between variations and tooth sizes

When tooth sizes and nucleotide variations of *PAX9* and *MSX1* were compared, it was found that 665 C-ins., 687 C-ins., 1451 G-ins., 1509 A-ins. in *PAX9* may statistically reduce or increase tooth sizes. Details are shown in Table 5. Any relation was not detected for *MSX1* mutations.

Discussion

Tooth development involves a complex series of genetic interactions involving growth factors, transcription factors, signal receptors and diffusible morphogens that interact within independent signaling pathways (33).

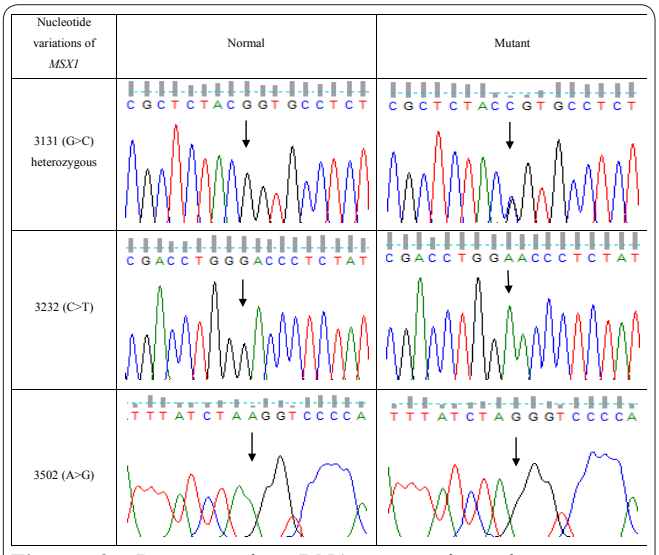

Figure 2. Representative DNA sequencing chromatograms showing mutations/polymorphisms of *MSX1*.

Table 4. Information of detected nucleotide variations in *PAX9* and *MSX1* and comparision of them between groups.

Gene names, nucleotide positions and variations	Region	Amino acid change	Control group (n=30)			Patient Group (n=31)			p values	Reported in pubmed and other databases
			Wild type	Hom. mut.	Het. mut.	Wild type	Hom. mut.	Het. mut.		
PAX9										
665 C-ins	Intron 1	-	30	0	0	17	14	0	**p<0,001***	**new**
679 G-del.	Intron 1	-	30	0	0	30	1	0	P=1	**new**
687 C-ins.	Intron 1	-	29	1	0	20	11	0	**p=0,003***	**new**
699 G>C	Intron 1	-	11	17	2	5	22	4	p=0,17	known (rs12883298)
704 C-ins.	Intron 1	-	30	0	0	30	1	0	p=1	**new**
719 C-ins.	Intron 1	-	30	0	0	29	2	0	p=0.49	**new**
725 C-del.	Intron 1	-	30	0	0	30	1	0	p=1	**new**
726 G>A	Intron 1	-	29	1	0	28	1	2	p=0.37	known (rs17104895)
726 C-ins.	Intron 1	-	30	0	0	30	1	0	p=1	**new**
744 C-ins.	Intron 1	-	30	0	0	30	1	0	p=1	**new**
754 A>G	Intron 1	-	7	6	17	3	18	10	**p=0.009***	**new**
767 A>G	Intron 1	-	21	1	8	15	1	15	p=.,21	**new**
800 T del.	Intron 1	-	30	0	0	30	1	0	p=1	**new**
836 C-ins.	Exon 2	Codon 11 frameshift-silent mut.	30	0	0	30	1	0	p=1	**new**
1409 G-ins.	Exon 2	Codon 202 silent mut.	30	0	0	29	2	0	p=0.49	**new**
1437 G-ins.	Intron 2	-	30	0	0	29	2	0	p=0.49	**new**
1451 G-ins.	Intron 2	-	30	0	0	25	6	0	**p=0.024***	**new**
1454 C-ins.	Intron 2	-	30	0	0	30	1	0	p=1	**new**
1475 G>A	Intron 2	-	27	3	0	19	12	0	**p=0.016***	known (rs2236007)
1476 C-ins.	Intron 2	-	30	0	0	30	1	0	p=1	known (rs752059786)
1509 A-ins.	Intron 2	-	30	0	0	23	8	0	**p=0.005***	**new**
1510 G-ins.	Intron 2	-	30	0	0	28	3	0	p=0.24	**new**
MSX1										
3131 G>C	Exon 2	G273R	30	0	0	30	0	1	p=1	**new**
3232 C>T	3´UTR	-	29	1	0	21	7	3	**p=0.012***	known (rs8670)
3249 T>G	3´UTR	-	30	0	0	30	0	1	p=1	**new**
3310 C>G	3´UTR	-	30	0	0	30	0	1	p=1	**new**
3502 A>G	3´UTR	-	28	1	1	21	8	2	**p=0.034***	known (rs12532)
3503 G-ins.	3´UTR	-	30	0	0	29	2	0	p=0.49	**new**
3508 C-del.	3´UTR	-	30	0	0	30	1	0	p=1	**new**

Table 5. Statistically significant associations between nucleotide variations and tooth sizes in *PAX9*.

Gene names and nucleotide variations	Tooth locations	Tooth sizes (mm) (Mean ± SD)			p values
			Mutation absent	Mutation present	
PAX9					
665 C-insertion					
	Maxillary Left	5	6,8 ± 0,67	7,16 ± 0,45	p=0,032*
		4	6,7 ± 0,53	7,38 ± 0,57	p=0.001*
		3	8,14 ± 0,61	7,78 ± 0,33	p=0.02*
		1	8,46 ± 0,67	7,93 ± 1,09	p=0.037*
	Maxillary Right	1	8,48 ± 0,66	8,14 ± 0,65	p=0.036*
		3	8,11 ± 0,58	7,7 ± 0,34	p=0.005*
		4	6,69 ± 0,55	7,15 ± 0,48	p=0.003*
	Mandibular Left	5	6,76 ± 0,67	7,26 ± 0,75	p=0.025*
		3	6,08 ± 0,65	6,41 ± 0,55	p=0.049*
	Mandibular Right	3	6,08 ± 0,64	6,49 ± 0,64	p=0.037*
		4	6,65 ± 0,54	7,16 ± 0,72	p=0.012*
687 C-insertion					
	Maxillary Left	4	6,71 ± 0,54	7,45 ± 0,53	p=0.001*
	Maxillary Right	4	6,71 ± 0,56	7,15 ± 0,47	p=0.008*
	Mandibular Left	5	6,76 ± 0,67	7,33 ± 0,72	p=0.025*
		3	6,07 ± 0,64	6,5 ± 0,53	p=0.02*
	Mandibular Right	2	5,54 ± 0,52	5,93 ± 0,47	p=0.022*
		3	6,08 ± 0,63	6,59 ± 0,62	p=0.017*
		4	6,63 ± 0,54	7,26 ± 0,65	p=0.004*
1451 G-insertion					
	Maxillary Left	3	8,14 ± 0,5	7,3 ± 0,74	p=0.007*
		1	8,5 ± 0,58	6,87 ± 1,15	p<0.001*
	Maxillary Right	1	8,5 ± 0,55	7,45 ± 0,94	p=0.002*
		3	8,1 ± 0,48	7,24 ± 0,6	p=0.001*
	Mandibular Left	1	5,34 ± 0,38	4,96 ± 0,06	p=0.01*
	Mandibular Right	1	5,35 ± 0,35	4,94 ± 0,12	p=0.004*
1509 A-insertion					
	Maxillary Left	4	6,77 ± 0,57	7,3 ± 0,71	p=0.026*
		3	8,15 ± 0,5	7,44 ± 0,69	p=0.006*
	Maxillary Right	3	8,12 ± 0,48	7,35 ± 0,58	p=0.001*

The majority of the mutations in hypodontia patients are located in the *PAX9* paired box domain (found in exon 2) and in the *MSX1* homeodomain (found in exon 2), both DNA binding regions.(31). Previous studies have demonstrated the important roles of PAX9 and MSX1 in tooth development and suggested that mutations in these genes were responsible for nonsyndromic tooth agenesis. Briefly, G6R (34), L21P (12), A26T (13), S43K (34), R59X (35), I87F (36), K91E (12), Q145X (37), Y160X (38), A168G (22), G22RfsX168 (39), R59fsX177 (12), A240P (40), G73fsX316 (9), V265fsX316 (11), R28P (16), R47W (7),G51S (14) K114X (10) were detected in exon 2 of *PAX9* whereas Q187X (15), A194V (1), R196P (8) A219T (41), A221E (42) mutations were detected in exon 2 of *MSX1*. These findings had been inspired and supported by animal studies showing that *MSX1* and *PAX9* are co-expressed in dental mesenchyme during the early stages of tooth development, and that homozygous deletion of *MSX1* or *PAX9* results in an arrest of tooth development at the bud stage (43-45). In our study, twenty two variations were detected in *PAX9* in which 18 of them are novel. In addition, 7 variations were found in *MSX1* in which 5 of them are novel and one of them cause amino acid change. When variations were compared between groups 665 C-ins., 687 C-ins., 754 A>G, 1451 G-ins., 1475 G>A, 1509 A-ins. in *PAX9*; 3232 C>T and 3502 A>G in *MSX1* were found statistically significant in patients (Table 4). Therefore it was suggested that, they may be associated with hyodontia, but further studies on the structure and functional significance of the genes are needed to establish the association between the *PAX9* and *MSX1* genotype and hypodontia prevalence.

There is a wide variation in the location and number of affected teeth, but most frequently lacking teeth are molars, second premolars, followed by lateral incisors (24, 46). In our patient group, lateral incisors are the most frequently missing teeth, followed by the second premolars, first premolars, first molars and the central incisors (Table 2). It has been shown that the *MSX1* mutations are associated with hypodontia that predominantly affects second premolars and third molars, whilst mutations in *PAX9* lead to agenesis of most molars, which can sometimes be combined with the absence of other teeth, including second premolars (47, 48). In our study, however mostly lateral incisors, first and second premolars are missing, any relation between mutatons and the type of congenitally missing teeth were not found.

Generally there is a wide variation in tooth size of patients. Reduced permanent tooth size is common in patients with hypodontia or oligodontia (49, 50). Similarly in our study when the tooth sizes of patients were compared with controls, some of the reduced tooth sizes were detected in patients. Contrary to this finding, some of the tooth sizes were found larger in patients than controls. Details are shown in Table 3. There are also few studies which investigate the relation between detected mutations and tooth sizes. Nieminen et al. found that K114X mutation in *PAX9* cause the reduction in size of some of the teeth (10). In our study we noticed smaller or larger tooth size in patients which may related with some detected mutations. Our results indicate that change of tooth size associated with tooth agenesis may be caused by 665 C-ins., 687 C-ins., 1451 G-ins., 1509 A-ins. in *PAX9*. Any relation was not detected for *MSX1* mutations. Statistically significant associations between nucleotide variations and tooth sizes in *PAX9* are shown in Table 5.

In conclusion, the present study has shown that *MSX1* and *PAX9* polymorphisms/mutations are associated with hypodontia and tooth sizes. Further studies are needed to establish the presence or absence of an association between these mutations and the PAX9, MSX1 phenotypes. Detailed knowledge of the aetiology of hypodontia may help to develop novel strategies in the prediction and prevention of this abnormality, which often requires expensive prosthetic, orthodontic and surgical treatments.

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