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Mutations in *MSX*1, *PAX*9 and *MMP*20 genes in Saudi Arabian patients with tooth agenesis

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ABSTRACT

Tooth agenesis in human being is the most common congenital anomaly associated with dental development. Mutations in many genes such as MSH homeobox 1 (*MSX1*), paired box gene 9 (*PAX9*), ectodysplasin A (*EDA*) and EDA receptor (*EDAR*) have been associated with familial form of this condition. However, in large majority of patients, genetic cause could not be identified. The primary aim of present study was to identify the causative mutation(s) in these genes in Saudi Arabian families diagnosed with non-syndromic form of disease. Direct sequencing of coding regions, including exon-intron boundaries of these genes was carried out. All identified nucleotide variations were also tested to exclude possibility of being rare polymorphisms. The sequence analysis of exons and exon-intronic regions of these genes revealed five new mutations that include four in *MSX1*, one in *PAX9* and one single nucleotide polymorphism (SNP) in majority of the patients in *MMP20*. One novel mutation in exon 1 of *MSX1* gene (5354C > G; A40G) was found in three patients. In addition, another novel mutation was detected in two patients in exon 3 (*PAX9*) as g.10672A > T which changes asparagine to isoleucine at position 40. These mutations were not found in any of the control subjects. A single SNP in *MMP20* genes (g.5066A > C) that changes lysine to threonine at position 18 was found in 10% controls as well. Our results for the first time demonstrates that mutations in *MSX1* gene might play an important role in hypodontia cases involving pre-molars and is a risk factor for this ethnic population mainly of Arabs and is first report linking these mutations with tooth agenesis.

1. Introduction

Human dentition consists of many types and shapes of permanent and deciduous teeth which are important part of the digestive system. Over 300 genes regulating tooth development has been identified so far (Balic and Thesleff, 2015). Teeth development, a highly coordinated and complex process is strictly regulated genetically via large number of molecules organized in signaling networks (Prasad et al., 2016). These molecules start a series of reciprocal interactions between the epithelium of oral cavity and its underlying mesenchyme and any disruption in these interactions could result in anomalies either in the number, size, morphology,

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and/or cyto-differentiation of teeth (Balic and Thesleff, 2015). Mutation(s) in these genes and their up- or down-regulation can affect tooth development. Genetic and environmental factors can also affect odontogenesis process thereby causing abnormalities with regard to number, size, shape, structure, eruption time and color of teeth (Galluccio et al., 2012). Hypodontia defined as absence of one to six permanent teeth is the most common dental anomaly affecting nearly 20% of world's population (De Coster et al., 2009). Excluding third molars, the prevalence of congenitally missing teeth varies from 3 to 12% in different populations (Polder et al., 2004). Tooth agenesis is classified either as non-syndromic or syndromic usually having other inherited abnormalities.

Molecular genetics studies carried out in various populations and countries have revealed the association of congenital lack of permanent teeth with several mutated genes involved in teeth development (Balic and Thesleff, 2015). In last couple of decades, mutations in many genes have been found to be associated with non-syndromic form of tooth agenesis viz. *MSX1* (Vastardis et al., 1996; Jumlongras et al., 2001; Liang et al., 2012; Kimura et al., 2013; Yamaguchi et al., 2014), *PAX9* (Das et al., 2002; Bianch et al., 2007), *AXIN2* (Yue et al., 2016), *MMP1* and *MMP20* (Kuchler et al., 2011), *FGF3* (Vieira et al., 2013), *TGFA* (Vieira et al., 2004), *EDA* (Ruiz-Heiland et al., 2016) and *WNT10A* (van den Boogaard et al., 2012). Recently, DNA methylation patterns have also been found to be altered in genes associated with development of cartilage, bone, teeth, and neural transduction which might provide insights into developmental biology and the pathobiology of this disorder (Wang et al., 2016).

MSX1-a homeobox protein localized on chromosome 4 is expressed in several embryonic tissues and is involved in several epithelial-mesenchymal interactions during tooth development. *PAX9* is a member of the PAX family of genes and is localized on chromosome 14. It is a paired domain transcription factor and plays an important role in cranio-facial development. *MSX1* a paired domain is related with the regulation of tooth shape and position where as *PAX9*has been suggested to establish the moment and the place of the odontogenesis start (Zhao et al., 2007). *MMP20*(enamelysin)localized on 11q22.3-q23 is expressed more or less exclusively by tooth-forming cells. It is well established that *MMP20* has an important role during enamel development and is involved at the cleavage and removal of most of the protein components of the extracellular enamel matrix (Shin et al., 2014). The diagrammatic gene structure of these genes is shown in Fig. 1.

Until now studies performed in different populations could not pinpoint or associate the pathogenic mutation(s) in genes involved in teeth development, lead to the argument that either other genes and/or yet to be discovered new mutations in other populations may be responsible for this condition which lead us to explore this idea. To our knowledge, no reports are available in the literature with regard to the mutation in key genes and their association with non-syndromic tooth agenesis in Saudi Arabian population. Therefore, the present study is carried out to detect mutations responsible for this condition in three most common genes viz. *MSX1*, *PAX9* and *MMP20* that play an important role in dental development among Saudi Arabian patients.

2. Materials & methods

2.1. Patients

All patients were recruited as research patients at College of Dentistry, King Saud University, Riyadh, Saudi Arabia and these patients were referred to the clinic because of requiring dental treatment. All patients or their families (In case children under age 15yr) were informed of the purpose and procedures of the research, and those who signed the informed consent form were selected to take part in this study. The study was conducted according to the principles expressed in the Declaration of Helsinki and Divisional Ethics Committee (DEC), College of Dentistry, King Saud University, Riyadh, Saudi Arabia has approved the study. Diagnosis of tooth agenesis was confirmed by clinician conducting thorough clinical and radiographic examination. The patients with diagnosis or suspected syndrome were excluded from this study. Genomic DNA was isolated from peripheral blood samples using Qiagen DNA mini kit (Qiagen). Forty one patients distributed among twenty one families took part in this project. Tooth agenesis in family members was initially reported by the patient, and congenital absence of one or more teeth were later on confirmed by the clinicians. In addition, 100 unrelated healthy individuals having complete permanent dentition were also included as control group.

2.2. Mutation analysis of MSX1, PAX9 and MMP20

PCR analyses of both exons of *MSX1*gene (NG_008121.1) were performed using KAPA2G Robust HotStart kit (Kappa Biosystems) in the presence of 5% DMSO because of high G + C content in the nucleotide structure of the *MSX1* and *PAX9* gene. The conditions for PCR were: 95 °C for 5 min; 45 cycles at 95 °C for 1 min and 58 °C for 45 s, 72 °C for 1 min followed by final extension at 72 °C for 5 min.

For all exons of *PAX9*gene (NG_013357.1), PCR amplifications were carried out using the KAPA2G Fast HotStartReadyMix (Kappa Biosystems, Wilmington, MA, USA). For amplification of exon 2 of *PAX9* gene, 5% dimethyl sulphoxide (DMSO) was also added to the master mix. The conditions for PCR were: 95 °C for 5 min; 40 cycles at 95 °C for 30 s and 58 °C (exon 1 and 3) and 54 °C (exon 2) for 45 s; and 72 °C for 1 min followed by a final extension at 72 °C for 5 min.

For all exons of *MMP20* gene(NG_012151.1), PCR amplifications were carried out using the KAPA2G Fast HotStartReadyMix (Kappa Biosystems, Wilmington, MA, USA). The conditions for PCR were: 95 °C for 5 min; 40 cycles at 95 °C for 30 s and 53 °C for 40 s for exons (1, 3, 4) or 55 °C for 40 s for exons (2, 5–8, 10) while 60 °C for 40 s for exon 9; and 72 °C for 45 s followed by a final extension at 72 °C for 7 min.

Mutation screening of the coding regions, including exon-intron boundaries of *MSX1*, *PAX9 and MMP20* genes was performed by bi-directional Sanger sequencing using ABI Prism BigDye Terminator Cycle Sequencing kit and ABI Prism 3730 capillary sequencer (Applied Biosystems, Foster City, CA, USA). Re-sequencing using forward and reverse primers was performed to confirm the presence of a novel mutation(s). To exclude the possibility if these novel mutations in *MSX1*, *PAX9* and *MMP20* genes might be a common polymorphism, 100 controls from the same region were also tested for the presence of these nucleotide variant. Chromatograms were aligned and compared to the reference sequences (as mentioned in previous sections).

All unclassified missense variants were further subjected to bioinformatics analysis using SIFT (http://sift.jcvi.org) and PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/index.shtml) modelling software. Predicted missense variants were also subject to Clustal alignment for the analysis of conserved residues. In this work, the term "mutation" is used when the gene-variant cause missense or frameshift predicted to be "damaging" or "potentially damaging" by PolyPhen. All variants were compared with sequences in NCBI (http://www.ncbi.nlm.nih.gov/Genbank/), Human Gene Mutation (http://www.hgmd.cf.ac.uk/ac/index.php), Biobase (http://www.biobase-international.com/) databases to exclude the possibility of chance polymorphisms.

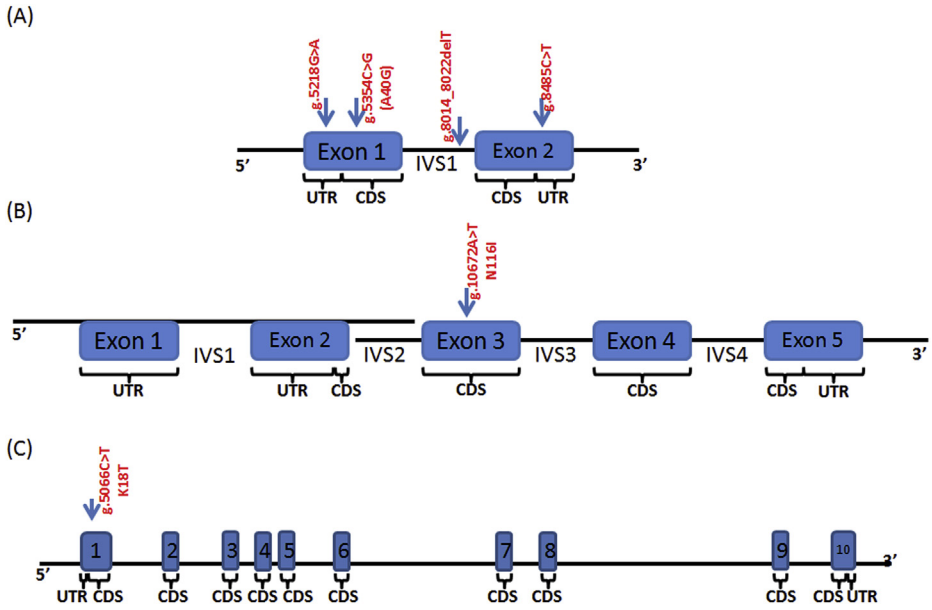

Fig. 1. Localization of mutations and polymorphisms in the MSH homeobox 1 (*MSX1*), paired box 9 (*PAX9*) and matrix metalloproteinase 20 or enamelysin (*MMP20*) genes detected in the current study. CDS: coding sequence; UTR: untranslated region and IVS (intron).

3. Results

In the present study, investigations were carried out on 21 families having patients affected with non-syndromic form of tooth agenesis. Only two of the current study cohort fulfilled the definition of oligodontia and the mean number of missing permanent teeth excluding the third molars in this group was 7 (range 6–8). Mutations detected in selected genes are represented diagrammatically (Fig. 1). Diagrammatic representations of missing teeth in each patient carrying mutation(s) are provided as dentograms (Fig. 2). During this study, the mutational analysis revealed suggested causative mutations only in 22 patients. Most of the mutations were found to be novel. The mutations are described in detail in Table 1 along with their genotypes and phenotypes. In nine patients, mutations were detected in multiple genes (Table 1). We didn't find any mutation in remaining patients.

In *MSX1*gene, the mutation, g.5354C > G (g.4860018C > G; rs36059701), which replaces alanine with glycine (p.Ala40Gly; Fig. 3A, B) was found in three patients. This missense mutation was interpreted as neutral using SIFT and benign with PolyPhen modelling. This mutation occurred simultaneously with another mutation at position g.8014_8022delT (g.4862686(delT); rs397878173; Fig. 3C, D). However, it was not possible to pinpoint the position of T as there are nine thymine residues are repeated.

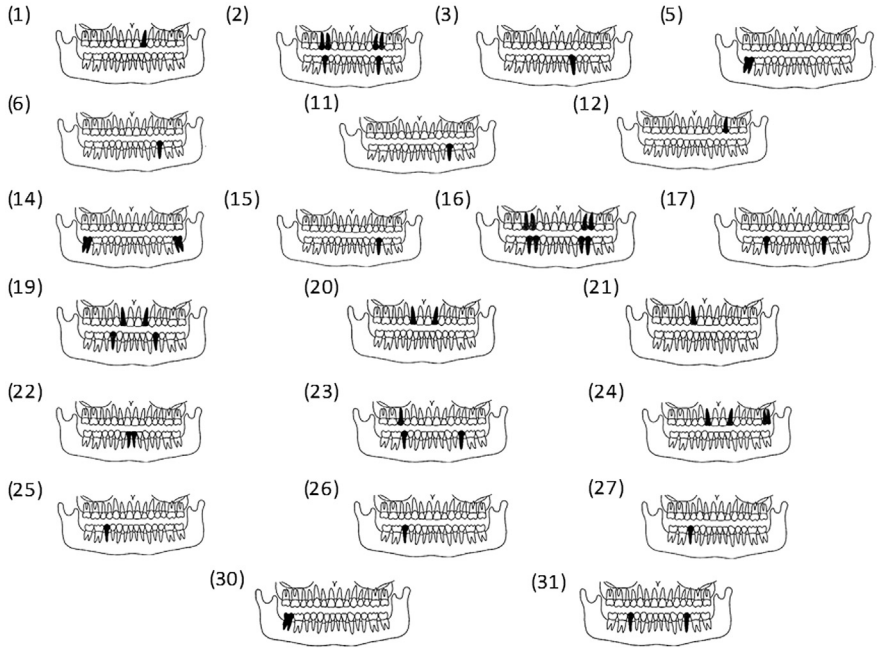

Fig. 2. Dentograms showing missing teeth (indicated in full black color) in patients showing mutations in *MSX1*, *PAX9* and SNP in *MMP20* gene.

Table 1
Patient details with missing tooth details and mutation in key teeth development genes.

S. No.	Patient no.	Age (yr)	Gender	Genotype/mutation	Jaw	R 8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	L 8
1	3[b,c]	7	F	MSX1:	U																
				g.5218G > A 5′ UTR	L											*					
2	31	17	F	MSX1:	U																
				g.5218G > A 5′ UTR	L				*									*			
3	1[b,c]	22	F	MSX1:	U										*						
				g.5354C > G (p.A40G)	L																
4	11[b,c]	50	F	MSX1:	U																
				g.5354C > G (p.A40G)	L													*			
5	14	24	M	MSX1:	U																
				g.5354C > G (p.A40G)	L		*													*	
1	1[b,c]	22	F	MSX1:	U										*						
				g.8014_8022 delT (intron1)	L																
				g.8485C > T 3′ UTR																	
2	2	11	F	MSX1:	U				*	*							*	*			
				g.8014_8022 delT (intron1)	L				*									*			
3	3[b,c]	7	F	MSX1:	U																
				g.8014_8022 delT (intron1)	L											*					
				g.8485C > T 3′ UTR																	
4	5[b]	21	F	MSX1:	U																
				g.8485C > T 3′ UTR	L		*														
5	11[b,c]	50	F	MSX1:	U																
				g.8485C > T 3′ UTR	L													*			
6	16[e]	15	F	MSX1:	U				*	*							*	*			
				g.8485C > T 3′ UTR	L				*	*							*	*			
7	17[b]	17	F	MSX1:	U																
				c.8485C > T 3′ UTR	L				*									*			
8	23	15	F	MSX1:	U				*												
				g.8485C > T 3′ UTR	L				*									*			
9	25[b]	14	F	MSX1:	U																
				g.8485C > T 3′ UTR	L				*												
10	30	25	M	MSX1:	U																
				g.8485C > T 3′ UTR	L		*														
1	21	18	M	PAX9:	U							*									
				g.10672A > T[a] (p.N116I)	L																
2	27	24	F	PAX9:	U																
				g.10672A > T[a] (p.N116I)	L				*												
1	5[b]	21	F	MMP20:	U																
				g.5066A > C (p.K18T)	L		*														
2	6[e]	50	F	MMP20:	U																
				g.5066A > C (p.K18T)	L													*			
3	11[b]	50	F	MMP20:	U																
				g.5066A > C (p.K18T)	L													*			
4	12	15	F	MMP20:	U													*			
				g.5066A > C (p.K18T)	L																
5	14	24	M	MMP20:	U																
				g.5066A > C (p.K18T)	L		*													*	
6	15	13	F	MMP20:	U																
				g.5066A > C (p.K18T)	L													*			
7	17[b]	17	F	MMP20:	U																
				g.5066A > C (p.K18T)	L				*									*			
8	19[e]	34	F	MMP20:	U							*			*						
				g.5066A > C (p.K18T)	L					*							*				
9	20	7	M	MMP20:	U							*			*						
				g.5066A > C (p.K18T)	L																
10	22	11	F	MMP20:	U																
				g.5066A > C (p.K18T)	L								*	*							
11	23	15	F	MMP20:	U				*												
				g.5066A > C (p.K18T)	L				*									*			
12	24	25	F	MMP20:	U							*			*					*	
				g.5066A > C (p.K18T)	L																
13	25[b]	14	F	MMP20:	U																
				g.5066A > C (p.K18T)	L				*												
14	26[d]	23	F	MMP20:	U																
				g.5066A > C (p.K18T)	L				*												
15	30[b]	25	M	MMP20:	U																
				g.5066A > C (p.K18T)	L		*														
16	31[b]	17	F	MMP20:	U																
				g.5066A > C (p.K18T)	L				*									*			

*: missing teeth.
[a] Deleterious (SIFT) and potentially damaging (PolyPhen-2).
[b] Mutations detected in multiple genes.
[c] Mutations in multiple locations within one gene; U: Upper jaw; L: Lower jaw; patient 1 and 14 siblings; patient 17 and 25 siblings; patient 5 and 11 daughter and mother.
[d] Enamel score:1.
[e] positive family history.

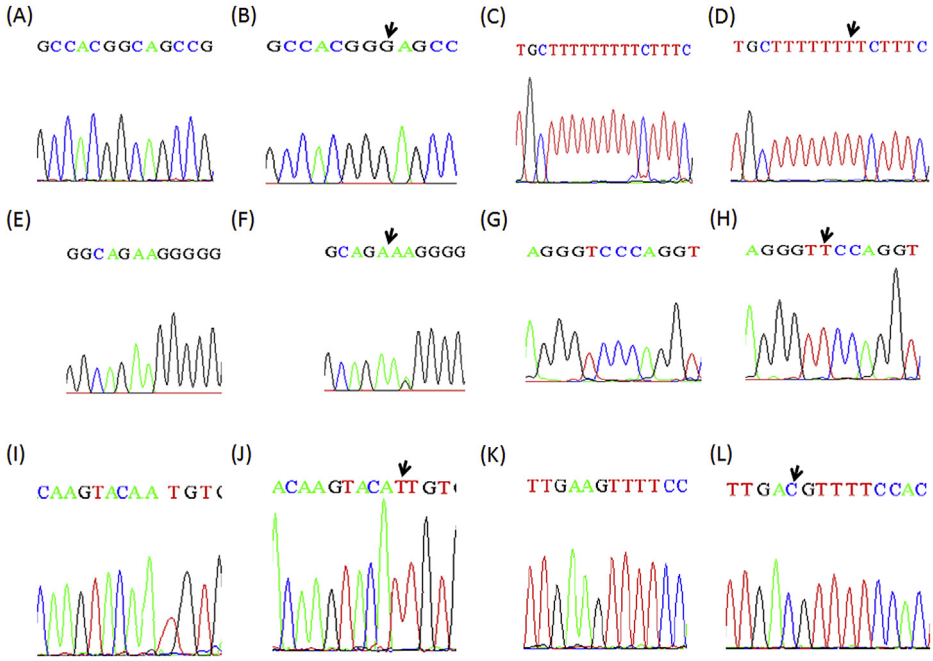

Fig. 3. Partial chromatograms showing mutations in different genes. (A) Exon 1 *MSX1* gene control, (B) Exon 1 *MSX1* gene patient showing g.5354C > G mutation; (C) Intron 1 *MSX1* gene control, (D) Intron 1 *MSX1* gene patient showing mutation g.8014_8022delT; (E) 5′ UTR region *MSX1* gene control, (F) 5′ UTR region exon 1 *MSX1* gene showing mutation g.5218G > A; (G) 3′ UTR region exon 2 *MSX1* gene control, (H) 3′ UTR region exon 2 *MSX1* gene showing mutation g.8485C > T; (I) Exon 3 *PAX9* gene control, (J) Exon 3 *PAX9* gene patient showing mutation g.10672A > T; (K) Exon 1 *MMP20* gene control, (L) Exon 1 *MMP20* gene patient showing mutation g.5066A > C.

This mutation didn't change any amino acid as it is present in intron 1. In addition, we also found two other mutations: g.5218G > A (g.4859872G > A) and g.8485C > T (g.4863149C > T; rs8670) respectively in 5′ and 3′ UTR region of the exon 1 and 2 (Fig. 3 E, F, G, and H). When we analysed the impact of this mutation on protein function using PolyPhen-2, it demonstrated it being benign and virtually have no impact on amino acid sequence of protein and are not directly involved in gene expression.

One novel mutation was identified in the highly conserved paired box sequence of *PAX9* gene (exon 3). This A > T (g.10672A > T) mutation replaces a polar amino acid asparagine with non-polar isoleucine (p.Asn16Ile). The novel *PAX9* mutation was not identified in any of the healthy controls. The affected patients lacked just one permanent tooth (Table 1; Fig. 3I, J). However, both these patients didn't have a positive family history of tooth agenesis. The SIFT prediction based on the degree of conservation of amino acid residues in sequence alignments derived from closely related sequences interpreted this missense mutation to be deleterious (SIFT proven score of −8.661). PolyPhen-2 analysis indicates that this mutation is probably damaging to the protein function with a score of 0.999 (sensitivity: 0.14; specificity: 0.999). No other mutation was found in any of the patients at any other position of *PAX9* gene.

In *MMP20* gene, we sequenced all ten exons and exon-intron boundaries. We found only one mutation in exon 1 as g.5066A > C (g.102625267A > C; rs2245803; p.Lys18Thr; c.66A > C; Fig. 3K, L) which resulted change in a positively charged amino acid lysine at position 18 to polar amino acid threonine (K18T). This mutation has been detected in many patients. This mutation has also been found in 10% controls, however, at this point of time no data about its incidence is available in Saudi Arabian population. The mutation is present in the signal peptide of the protein. We didn't find any other mutation in any of the other exons.

ClustalW alignments in different proteins show that most of the residues are highly conserved in various species (Fig. 4).

In total, 46 teeth were missing in 22 patients with atleast one mutation in any of these genes. Of these missing teeth, 28 (%) are maxillary teeth and 18 (%) are mandible teeth. The number of missing teeth in individuals with *MSX1*, *PAX9* and *MMP20*mutations varied from 1 to 8. Premolars were most commonly missing, followed by molars. Table 2 lists the number of patients with number of missing teeth. Most of these mutations (and SNP in MMP20) were found to be bi-allelic in nature except 5′-UTR region in exon 1 of MSX1 gene (g.5218G > A; Table 2). None of the two oligodontia patients reported any abnormalities in nails, hair, skin and sweat glands and didn't show any symptoms of ectodermal dysplasia. We also examined the enamel score in all patients especially those carrying mutations in *MMP*20 gene showed normal enamel while one patient (#26) showed a slight aberration from the translucency of normal enamel ranging from a few white flecks to occasional white spots.

4. Discussion

In the present study, all exons and exon-intron boundaries of *MSX1*, *PAX9* and *MMP20* genes were sequenced and analysed for the presence of any novel mutations and polymorphisms.

Four mutations detected in *MSX1* genes are novel. Previous reports found the association of mutations in *MSX1* gene with missing second pre-molar and third molars (Vastardis et al., 1996). However, in the present study, even though it is mainly the missing premolar but in some patients molars, incisors and canines are also found missing (Bergendal et al., 2011). *MSX1* mutations are often related with the absence of pre-molars where as *PAX9* mutations tend to be associated with molars (reviewed in Wang et al., 2011). *PAX9* mutations have been reported to be associated with the loss of all/any type of permanent teeth likely due to its phenotypic effects in relation to dosage relationship (Kapadia et al., 2006). Most of the mutations found in the present study are associated with the loss of pre-molars. C > G transition mutation causes the

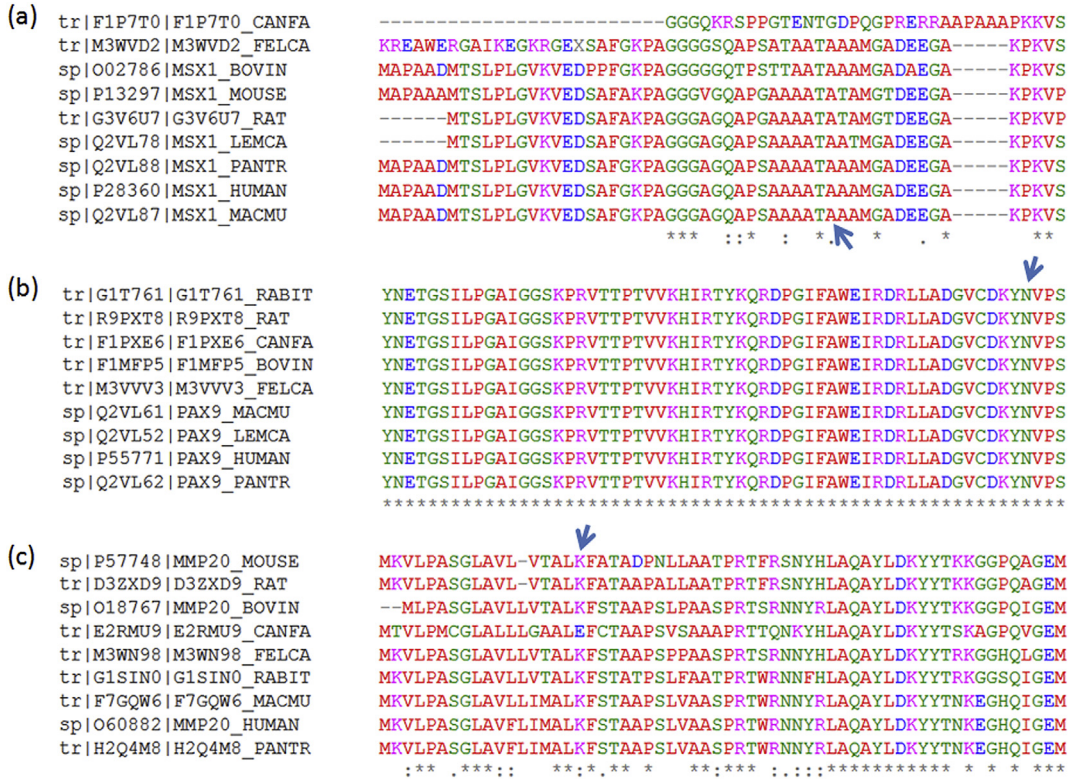

Fig. 4. ClustalW multiple alignment of MSX1, PAX9 and MMP20 protein sequences of *Homo sapiens*, *Mus musculus*, *Lemur catta*, *Pan troglodytes*, Rattusnorvegicus, Bostaurus, Feliscatus, Oryctolaguscuniculus, Macacamulatta and Canisfamiliaris. The position of the missense mutations resulting in exchange of an evolutionary conserved amino acid residue is marked with arrow. Amino acids indicated as AVFPMILW are shown in red, DE are blue, RK are magenta, STYHNGQ are green and all other residues are grey. The stars, dots and colons below the alignment indicate degree of conservation in the columns, (a) MSX1, (b) PAX9 and (c) MMP20. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Distribution of mutations in each gene with number of missing teeth and whether the mutation mono- or bi-allelic.

Gene	No of missing teeth Hypodontia/Oligodontia (O)	No of patients	Mono-/Bi-allelic
MSX1	1	3	2/1
	2	2	0/2
PAX9	1	8	0/8
	2	1	0/1
	3	1	0/1
	6 (O)	1	0/1
	8 (O)	1	0/1
MMP20	1	8	0/8
	2	5	0/5
	3	2	0/2
	4	1	0/1

substitution (A40G) of a highly conserved residue in *MSX1* protein. Therefore, it is highly likely it may alter protein-protein interactions involving *PAX9* protein. Recently, a frameshift mutation involved with tooth agenesis specifically associated with premolar and third molar was reported (Al Fawaj et al., 2015). Two mutations in *MSX1* gene were found located in the 5′ and 3′-untranslated region (5′-UTR and 3′-UTR)), which may influence the processes such as transcription, post-transcriptional RNA modifications, translation (Wang et al., 2005) or microRNA functions (Cao et al., 2010). Most patients suffered from agenesis of the second premolars or canine. Therefore, we could not decide if these mutations represent just inter-individual variation without having significant consequences. No mutations in *MSX1* coding region have been found in patients with hypodontia phenotypes, and it has been suggested that a multi-genic inheritance may be considered a likely aetiology for this condition (Boeira Junior and Echeverrigaray, 2012).

Both coding and non-coding regions of *PAX9* gene are highly conserved evolutionary. The mutation N116I located in the coding region possibly contributes to both structural and functional changes in the protein. ClustalW alignment provided this clue of being this amino acid highly conserved in mammals. It is highly likely that this change in protein structure may influence its spatial structure, three dimensional folding and thermostability. These changes have the potential to disturbing its normal functionalities like DNA-protein binding and its interactions with transcriptional factors (Gerits et al., 2006). Presence of hypodontia indicates the developmental tooth anomaly that constitutes one or more missing teeth where as oligodontia is described as missing ≥6 teeth. Therefore, the later represents a more severe clinical entity of tooth agenesis. In the present study we found both hypo- and oligodontia

in one family that indicate variable expression of shared genetic factors as shown previously (Bergendal et al., 2011). So far, fifteen mutations have been reported that are associated with non-syndromic form of tooth agenesis and majority of these are present in highly conserved region critical for MSX1 transcription. Though the mutation(s) detected in the present study are not within this region, however it still have influenced gene-gene or gene-protein interactions during the odontogenesis.

MSX1 is widely expressed at sites with epithelial-mesenchymal interactions during the bud and cap stage of odontogenesis (Balic and Thesleff, 2015). It has already been reported that *MSX1* mutations are associated with sporadic form of tooth agenesis as well as with cleft-lip/palate (Tongkobpetch et al., 2006). More than two third of these mutations associated with sporadic form of tooth agenesis are in the homeodomain whereas mutations reported in cleft/lip/palate all lie outside of homeodomain. This observation provides evidence that mutations at different sites of *MSX1* gene have varying effects with regard to orofacial development leading to variable phenotype. However, the exact mechanism how this happens still remains elusive.

Till date thirteen of the thirty mutations reported in *PAX9* gene associated with severe form of tooth agenesis are located in the paired-box domain (Tallón-Walton et al., 2014; Liang et al., 2016). N116I mutation (present case) is the first reported mutation in exon 3. The increasingly high incidence of tooth agenesis in the last few decades coupled with relatively small number of mutations in various genes associated with tooth genesis provide evidence that it is a complex and heterogeneous trait. Mutations in multiple genes, altered protein-protein interactions as well as reduction in gene dosage could lead to specific phenotype (Vieira et al., 2004). In the present study we found double mutations in *MSX1* and *PAX9* genes in association with polymorphic variant in *MMP20* that are collectively contributing to hypo- and oligodontia in some patients. Gene-gene interaction analyses are becoming more common because there is growing evidence that these interactions are paramount to determine the susceptibility to common diseases. This might have resulted in potentially altered protein-protein interactions thereby causing functional changes for tooth development may be a risk factor for tooth agenesis. A recent report also provides some evidence in this direction (Mu et al., 2014). However, a large study cohort is required to substantiate these findings. It is now known that Pax9 is a transcriptional factor which regulates expression of other key odontogenic molecules in the mesenchyme during the odontogenesis at bud stage and enables the transition of tooth germ to next cup stage, and disruption of *PAX9* gene function causes inhibition of odontogenesis at the bud stage (Krejci et al., 2007). Exon 3 of PAX9 gene is of much significance because of its DNA-binding domain coded in this area allows the protein to act as a transcription factor and 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 this population and on the other hand, we detected polymorphisms not described previously in any of the studies. This discrepancy could be caused by a genetic difference of Saudi people with tooth agenesis versus previously studied populations (Gerits et al., 2006; Hlousková et al., 2015; Pawlowska et al., 2010; Zhao et al., 2007). Standard PAX9 nucleotide sequences also occurred in patients with confirmed dental agenesis, implying thus multi-factorial aetiology of this condition. Tooth development is controlled by expression of a large number of other genes not analysed in this study and can be a cause of tooth agenesis in our patients. In vivo studies have shown that mutated MSX1 and PAX9 proteins showed variations in their protein spatial structure, thus influencing their thermo-stability and/or 3-D folding, which might have disturbed the normal functional activities of the mutant protein and altered their DNA-binding capacity and interactions with other transcriptional factors. It is highly likely that occasionally, these proteins may completely lose their functionality (Gerits et al., 2006).

We found one intronic mutation 8014-8022delT in three patients. No other intronic mutation was found in any of the other genes in the remaining patients associated either with hypo- or oligodontia. However, multiple mutations were found in some patients involving *MSX1* and *PAX9* genes. This observation provides evidence that gene-gene and/or protein-protein interactions can lead to this type of phenotype. Presence of some intronic mutations in three unrelated patients either alone or in combination with mutations either in *MSX1* or *PAX9* gene provide evidence that odontogenesis is very complex process and gene interactions at multiple levels are necessary to better understand this developmental anomaly. Genotype-phenotype correlations show that missing premolar is associated with mono-allelic mutation on MSX1 gene. Similar findings have been were reported with regard to WNT10A mutations (Arzoo et al., 2013). The significance of intronic mutations in many genes are generally seen in the prospective of pre-mRNA splicing, which can either be canonical or alternative. It has been reported that *MSX1* gene is expressed by two different pathways employing two different START codons during the translation process, however, it could be excluded if there exits other form(s) of altered expression, perhaps including alternative splicing and it is quite clear that this process is highly regulated by specific enzymes. Not only 5′ and 3′ sequences of splicing sites, pyrimidine-rich tract and branch site that are considered essential for canonical splicing, may also play a crucial role in alternative splicing (Modrek and Lee, 2002). Therefore, linking of intronic mutations in *MSX1* gene with phenotypic consequences requires further larger studies, since it was not clear to us how alternative splicing of mRNA in this gene leads to this phenotype. As canonical splicing sites are short and frequently degenerated, additional cis-acting signals may be required to further guide splicing machinery away from potentially incorrect splicing sites towards proper splicing sites. It is possible that this intronic mutation in *MSX1* gene negatively affects pre-mRNA splicing (Pawlowska et al., 2010; Sery et al., 2015). Mouse model studies have shown that both *Msx1* and *Pax9* are co-expressed during craniofacial and tooth development. In homozygous animals, all teeth including first molar fail to develop primarily after bud formation (Peters et al., 1998). It has been shown that at this stage of tooth development, the expression of both these genes is necessary for mesenchymal expression of another gene *Bmp4* to progress to cap stage. Contrary to humans, in heterozygous mice, loss of function do not affect either tooth development or secondary palate this indicating the requirement of different gene dosages (Blin-Wakkach et al., 2001; Ogawa et al., 2006; Nakatomi et al., 2010). Changes in gene dosage have also been found to influence the size of tooth primordia during the initiation stage of tooth development. As both these genes are co-expressed in dental mesenchyme, their interaction directly or indirectly affect expression of various factors that also control the accurate size and duration of molecular response required in adjacent dental epithelium. It is highly likely that P*ax9*/*Msx1* interaction may be required to indirectly regulate Bmp4 protein stability or its efficient diffusion, both of which providing possible explanations for a spatially restricted response to Bmp4 signalling in the lower incisor epithelium of $Pax9^{+/-}$;$Msx1^{+/-}$ mutants (Nakatomi et al., 2010). Recently it has also been hypothesized that variable expressivity of Msx1may result either from varying genetic backgrounds associated with the risk of non-syndromic tooth agenesis or may be due to the differences between the MSX1 transcriptional regulation that occurs at the *myoD*-promoter and at other promoters related to

odontoblast differentiation such as bmp2, bmp4, and runx2, which have been previously shown to be suppressed by MSX1 (Petit et al., 2009; van den Boogaard et al., 2012; Feng et al., 2013).

In case of *MMP20* gene, the mutation detected in many patients lies in the first 22 amino acids that form the important part of the protein. It is known that first 22 amino acids together constitute a signal peptide containing a long stretch of hydrophobic amino acids. This will form a single alpha helix of polypeptide helping its translocation. Therefore, it is highly likely that the peptide's translocation is affected. Moreover, this signal peptide functions as a RNA element, and promotes nuclear mRNA export. In addition this can also result in altered polypeptide folding of the signal peptide. There is a possibility that this mutation in *MMP20* gene could result in the reduction in mRNA activity. This can impact on diminished *MMP20* activity resulting either partial or complete failure to process/turn over enamel protein during the secretary stage. Sixteen patients show polymorphic mutation and half of these patients also show mutations in other investigated genes. Therefore, it is possible that *MMP20* may itself not lead to this condition but this polymorphism may accelerate the pathogenic process due to disruption of gene-gene interactions during the developmental stages of tooth development. This could involve *MSX1* and *PAX9* and perhaps other genes such as *EDA*, *WNT10A* not selected for the present project.

Overall, it can be concluded that there exists a high degree of heterogeneity among hypo- and oligodontia and our findings don't suggest if a genotype-phenotype relationship exists in this cohort. It is also possible that either mutation in one gene at different locations or mutations in many genes may still be sufficient to explain this condition. Our results indicate that screening of *MSX1* and *PAX9* genes has identified one third patients having genetic cause of non-syndromic form of tooth agenesis in this population. To further elucidate future GWAS studies in larger cohort should be carried out that primarily focus in identifying novel loci will lead to better understand the molecular mechanisms involved with the odontogenic development (Stranger et al., 2011). In addition, these will also help in explaining ethnic variation as well as relationship between genetic variants and variable phenotype. Impact of epigenetic changes, post-transcriptional modulation and various environmental factors can also exert significant influence to manifest this condition. We also found a substantial overlap between mutant gene and pattern among missing teeth. Absence of genotype-phenotype relationship may be due to small number of patients. Even though consanguineous marriages are quite common in this population but we are unable to suggest if any of the identified mutations are associated with a high degree of penetrance when transmitted through generations.

Conflict of interest

None.

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