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Mutations in MSX1, PAX9 and MMP20 genes in Saudi Arabian patients with tooth agenesis

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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) a sg. 10672 A > 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.

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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,
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; Junilongras 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), FG23 (Vieira et al., 2013), TGFα (Vieira et al., 2004), EDN1 (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; 45 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 bidirectional 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.biobaseinternational.com/) databases to exclude the possibility of chance polymorphisms.
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.
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*: 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.
[e] positive family history.
With non-polar isoleucine (p.Asn16Ile). The novel mutation was not identified with a score of 0.999 (sensitivity: 0.14; specificity: 0.999). No other mutation was found in any of the other exons. Of these missing teeth, 28% are maxillary teeth and 18% are mandibular 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). The two oligodontia patients reported any abnormalities in nails, hair, skin and sweat glands and didn’t show any symptoms of ectodermal dysplasia. We examined the enamel score in all patients especially those carrying mutations in MMP20 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
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'-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 while 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

![Fig. 4. ClustalW multiple alignment of MSX1, PAX9 and MMP20 protein sequences of Homo sapiens, Mus musculus, Lemur catta, Pan troglodytes, Rattus norvegicus, Bostaurus, Felis catus, Oryctolagus cuniculus, Macaca mulatta and Canis familiaris. 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, STYHQG 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.)](image)

<table>
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<th>Table 2</th>
<th>Distribution of mutations in each gene with number of missing teeth and whether the mutation mono- or bi-allelic.</th>
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<td>Gene</td>
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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 (Tallon-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; Hlouskovà et al., 2015; Pawłowska 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 maxillary 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 (Pawłowska 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 Pax9/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 Msx1 may 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
protein. It is known that this can also result in altered polypeptide folding of the signal RNA element, and promotes nuclear mRNA export. In addition translocation is affected. Moreover, this signal peptide functions as heterogeneity among hypo- and oligodontia and our MSX1 developmental stages of tooth development. This could involve condition but this polymorphism may accelerate the pathogenic diminished could result in the reduction in mRNA activity. This can impact on these patients also show mutations in other investigated genes.


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