MMP1 and MMP20 contribute to tooth agenesis in humans

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ABSTRACT

Objective: Variations in genes that are critical for tooth formation may contribute to the tooth agenesis. MMPs are potential candidate genes for dental alterations based on the roles they play during embryogenesis. The aim of this study was to investigate the possible association between MMP1, MMP3, and MMP20 and tooth agenesis.

Methods: One hundred sixty-seven nuclear families from two different populations were analysed, 116 from Brazil and 51 from Turkey. Probands had at least one congenitally missing tooth. DNA samples were obtained from blood or saliva samples and genotyping was performed using TaqMan chemistry. In addition, Mmp20 was selected for quantitative real-time polymerase chain reaction analysis with SYBR Green I Dye in mouse tooth development.

Results: Associations between tooth agenesis and MMP1 ($p = 0.007$), and MMP20 ($p = 0.03$) were found in Brazilian families. In the total dataset, MMP20 continued to be associated with tooth agenesis ($p = 0.01$). Mmp20 was not expressed during the initial stages of tooth development.

Conclusion: Our findings provide evidence that MMP1 and MMP20 play a role in human tooth agenesis.

1. Introduction

Tooth agenesis, which is defined as congenital absence of one or more teeth, is the most common human developmental anomaly.[1] The incidence varies with tooth class. Reports on the overall prevalence of missing permanent teeth vary substantially from 2.6% to 11.3%, excluding third molars.[2–4]

Tooth agenesis can occur in association with other genetic diseases or as an independent trait. Non-syndromic tooth agenesis shows wide phenotypic heterogeneity and is classified as sporadic or familial.[5–8]

Evidence supporting a genetic aetiology for tooth agenesis is well established and genes implicated in epithelial–mesenchymal interactions serve as potential candidates. To

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Table 1 – Details on the genetic markers studied in families.				
Gene	Location in the gene[a]	SNP	Flanking sequence[b]	Locus
MMP1	Intron 2	rs470747	ATTTTCTGTAATGA[C/T]TTTCAGAGTGCAC	11q22–q23
MMP3[c]	Near 5′UTR	rs3025058	GGACAAGACATGG[-/T]TTTTTCCCCCCATC	11q23
MMP20	Intron 1	rs1784418	GCTATCCTTTCTGT[A/G]GGCACAGTCCTTT	11q22.3–q23

[a] Locations obtained from the UCSC Genome Browser on Human Mar. 2006 Assembly (http://genome.ucsc.edu).
[b] Flanking sequences obtained from ENTREZ SNP database (http://www.ncbi.nlm.nih.gov/sites/entrez).
[c] Alleles are commonly designated as 5A and 6A in the literature.

date, severe forms of tooth agenesis (oligodontia) have been linked to mutations or deletions in MSX1, PAX9, AXIN2, and EDA.[9] In most of these families, tooth agenesis is segregating in an autosomal dominant fashion. However, the origin of the most common forms of tooth agenesis (hypodontia) remains largely unknown.

Animal models, have contributed to the understanding of tooth development and dental alterations. Molecular studies of odontogenesis, using the mouse tooth as a model system, have indicated that tooth formation is regulated by interactions between epithelial and mesenchymal cells and requires protein products of a number of genes. Mutations in several of these genes can cause an alteration in tooth development.[6,7] In mice, matrix metalloproteinases are expressed in craniofacial structures, suggesting that the expression of these genes is critical for the early craniofacial development and development of the dentition.[10] Matrix metalloproteinases constitute an important family of zinc-dependent endopeptidases, which are able to degrade components of extracellular matrix.[11] Extracellular matrix plays an important role in mechanisms involved in tissue interactions that regulate tooth development.[12]

The aim of the present work was to investigate if genetic variation in MMP1, MMP3, and MMP20 is associated with isolated human tooth agenesis. In addition, we evaluated the expression of Mmp20 in mouse tooth development, since our results suggested this gene could be involved in tooth agenesis.

2. Materials and methods

This study was approved by the University of Pittsburgh Institutional Review Board (IRB), as well as the appropriate Ethics Committees at the Federal University of Rio de Janeiro, University of São Paulo, and Istanbul University. Appropriate informed consent was obtained from each family member.

The study group consisted of 167 nuclear families (father–mother-affected child) whose proband presented with at least one permanent tooth congenitally absent, with the exception of third molars. The patients were from two different populations, 116 were from Rio de Janeiro, Brazil, which is an admixed population of Europeans and Africans, with a very small percentage of Native South Americans. The second populations consisted of 51 trios from Istanbul, Turkey.

None of the families reported history for clefts and dental alterations were the sole disorder affecting these patients. Information regarding family history for tooth agenesis was obtained and positive family history was defined as any proband's relative with reported congenital tooth agenesis.

After informed consent was obtained, cheek swab, whole saliva, or whole blood DNA was obtained from family trios and extracted by modifications of published protocols.[13,14] The two populations were analysed independently and then in combination.

2.1. MMP1, MMP3, and MMP20 genotyping

Genetic polymorphisms in the MMP1, MMP3, and MMP20 were genotyped by real-time polymerase chain reactions using the Taqman method[15] in an ABI PRISM 7900 Sequence Detection System instrument (Applied Biosystems, Foster City, CA). Assays and reagents were also supplied by Applied Biosystems (Foster City, CA). Marker information is included in Table 1. The polymorphism in MMP3 was chosen because it was recently associated with isolated forms of cleft lip and palate.[16] The other two polymorphisms in MMP1 and MMP20 were chosen due to their location in the genes and frequency in populations of European origin.

Chi-square was used to test if the observed genotype frequencies were in Hardy–Weinberg equilibrium. The proband, father, and mother genotypes were compared to determine the transmitted alleles vs. the non-transmitted alleles. The family based association test software package was used to detect transmission distortion.[17] Significance was established for alpha lower than 0.05.

2.2. Animals and tissue collection and processing

Swiss mice were sacrificed at various stages of embryonary development (from E13 to E20) and at 1-day postnatal. Day 0 was defined according to the identification of a vaginal plug. The animals received food and water ad libitum and they were euthanized by a lethal dose of anaesthetics, in agreement with the Brazilian Federal Guidelines of Animal Experimentation. Mandibles (5 specimens per period) were dissected out using stereoscopic magnifying lens and embedded, immediately, in RNA stabilization solution (RNA layer, Ambion, Austin, TX).

2.3. Quantitative real-time polymerase chain reaction

Total RNA was extracted from homogenized tissues with TRIzol, according to the manufacturer's instructions, and RNA integrity of samples was evaluated based on the intensity of 28S and 18S rRNA bands in 1% agarose gels and on $A_{260/280}$ ratio between 1.8 and 2.0. Samples of RNA were reverse transcribed with Superscript III™ using oligo (dT) primers and RNaseOUT, after treatment with DNase I (all reagents from Invitrogen, Carlsbad, CA). Quantitative real-time polymerase chain reaction was carried out by an ABI PRISM 7500 Sequence Detection

Table 2 – Primers used for quantitative real-time polymerase chain reaction analysis.

Target gene	Accession number	Position (5′–3′)	Primer sequences (5′–3′)	Amplicon size (base pairs)
Mmp20	NM_013903	F: 325–344	F: tcctgatgtggctaactacc	129
		R: 434–453	R: gccatctgtattgccttgtc	
Hprt1	NM_013556	F: 274–293	F: tggacaggactgaaagactt	119
		R: 373–392	R: aatgtaatccagcaggtcag	
β-Actin	NM_007393	F: 209–228	F: atggtgggaatgggtcagaa	84
		R: 273–292	R: aatggggtacttcagggtca	
Gapdh	NM_008084	F: 146–164	F: cgaccccttcattgacctc	140
		R: 267–285	R: ctcgctcctggaagatggt	
Tubulin (Tubb2a)	NM_009450	F: 118–136	F: caaccagatcggcgctaag	133
		R: 231–250	R: gttgccagcagcttcattgt	

Note: F indicates forward; R indicates reverse.

System instrument with SYBR Green I Dye reagent (Applied Biosystems, Foster City, CA).

The gene-specific primer sets for Mmp20 and housekeeping genes (Table 2) were designed using the Gene Tool 2.0 software (Biotools Incorporated, Edmonton, Alberta, Canada). All quantitative real-time polymerase chain reactions were performed in a total volume of 25 μL, containing 2.5 μL of cDNA sample, 10 pmol of each primer (400 nM), and 12.5 μL of SYBR Green Master Mix® (Applied Biosystems, Foster City, CA). The thermal cycling was carried out by starting with one hold cycle of 95 °C for 10 min, followed by 40 amplification cycles of 95 °C for 10 s and 60 °C for 1 min. An E13 sample was used for calibration purposes.

Relative analysis was performed,[18] a mathematical model and polymerase chain reaction efficiencies were obtained from 5-fold serial dilutions of cDNA templates quantified in triplicates. The polymerase chain reaction efficiency of each gene assay was determined from the respective cDNA dilution versus Ct plots. The reaction efficiency was calculated using the equation $E = 10(-1/\text{slope})$ where 'E' is the efficiency and 'slope' is the gradient of the best fit line. Dissociation curve analysis was performed at the end of cycling to verify the specificity of the polymerase chain reaction product.

Normalized expression was obtained after expression stability measurement of the endogenous control genes tested (β-actin, Gapdh, Hprt1 and, tubulin). The GeNorm algorithm[19] was used to determine the normalization factor.

Statistical analysis was performed using one-way ANOVA and Bonferroni post-test. p-values lower than 0.05 were considered statistically significant and comparisons were made between all possible pairs. Values were analysed using

Table 3 – Characteristics of the study populations.

Population aspects	Brazilian (n = 116)	Turkish (n = 51)	Combined (n = 167)
Gender (%)			
Males	42 (36)	24 (47)	66(39.6)
Females	74 (64)	27 (53)	101(60.4)
Number of congenitally missing teeth (%)			
1	44 (38)	12 (25)	56(33.5)
2	46 (40)	17 (33)	63(37.7)
3–5	19 (16)	11 (21)	30 (18)
6 or more (oligodontia)	7 (6)	11 (21)	18 (10.8)
Other Characteristics (%)			
Positive family history	41 (35.3)	–	41(24.5)
Associated small lateral incisor[a]	13 (11.2)	–	13 (7.8)
Associated enamel hypoplasia	2(1.7)	–	2 (1.2)
Associated talon cusp	1 (0.9)	–	1 (0.6)
Type of teeth affected (%)			
Upper second premolar	36 (13.2)	8 (9.4)	44 (12.2)
Lower second premolar	68 (24.8)	29 (34.1)	97(27.0)
Upper lateral incisor	66 (24.1)	15 (17.6)	81 (22.6)
Lower incisors	32 (11.7)	12 (14.1)	44(12.2)
Upper first premolar	14 (5.1)	3 (3.5)	17(4.7)
Lower first premolar	12 (4.4)	1 (1.2)	13 (3.6)
Upper molar	15 (5.5)	–	15 (4.2)
Lower molar	20 (7.2)	6 (7.1)	26 (7.2)
Upper canines	8 (2.9)	6 (7.1)	14 (3.9)
Lower canines	3 (1.1)	4 (4.7)	7 (1.9)
Upper central incisor	–	1 (1.2)	1 (0.3)

[a] Small lateral incisor represents cases of peg-shaped teeth and microdontia in upper lateral incisors.

Table 4 – Summary of family based association test results.

Gene	SNP	Allele	Brazil			Turkey			Combined		
			S	E (S)	p-value	S	E (S)	p-Value	S	E (S)	p-Value
MMP1	rs470747	C	21.0	16.5	0.007	18.0	18.5	0.82	40.0	36.5	0.22
		T	1.0	5.5		16.0	15.0		18.0	21.5	
MMP3	rs3025058	5A	66.0	71.5	0.28	31.0	27.5	0.26	97.0	36.0	0.78
		6A	104.0	98.5		33.0	36.5		135.0	36.0	
MMP20	rs1784418	A	63.0	73.5	0.03	20.0	23.83	0.17	86.0	100.5	0.01
		G	73.0	62.5		30.0	26.17		104.0	89.5	

Notes: FBAT output variables: S = test statistic (i.e., genotypic distribution in the offspring conditioned on affection status and parental genotypes); E (S) = expected value for S.

Table 5 – Summary of MMP20 expression studies.

Bonferroni's multiple comparison test	Mean difference	95% Confidence interval of the difference	t	p-Value
E13 vs. E16	−0.1625	−3.756 to 3.431	0.2158	Not significant
E13 vs. E17	−5.566	−9.159 to −1.972	7.393	<0.05
E13 vs. E19	−11.23	−14.82 to −7.635	14.92	<0.0001
E13 vs. PN1	0.5511	−3.043 to 4.145	0.7320	Not significant
E16 vs. E17	−5.403	−8.997 to −1.810	7.177	<0.05
E16 vs. E19	−11.07	−14.66 to −7.473	14.70	<0.0001
E16 vs. PN1	0.7136	−2.880 to 4.307	0.9478	Not significant
E17 vs. E19	−5.663	−9.257 to −2.070	7.522	<0.05
E17 vs. PN1	6.117	2.523 to 9.711	8.125	<0.05
E19 vs. PN1	11.78	8.187 to 15.37	15.65	<0.0001

the statistical package GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA).

3. Results

The Brazilian dataset contains 71 sporadic cases and 45 familial cases. Seventy-four were females and 42 were males. Forty-one cases presented positive family history for tooth agenesis and 16 cases were associated with other tooth developmental alterations, such as hypoplastic enamel, peg-shaped upper lateral incisors, and microdontia. The Turkish dataset consisted of 51 trios. Twenty-six were females and 25 were males. All Turkish cases were of sporadic origin. The details about these two populations are presented in Table 2.

All SNPs showed Hardy–Weinberg equilibrium in both the affected probands and unaffected individuals. Association could be seen between tooth agenesis and MMP1 ($p = 0.007$) and MMP20 ($p = 0.03$) in families of Brazilian origin (Tables 3 and 4).

3.1. Expression of MMP20

Since the genetics analysis suggested MMP20 is involved with tooth agenesis, the expression of this gene was evaluated, in particular during early dental development. GeNorm ranked *Gapdh* and *Hprt*, respectively, as the more stable genes, and *β-actin* and *tubulin*, respectively, as the less stable ones. However, because internal control gene-stability measurement (M) was appropriated for all housekeeping genes studied ($M < 1.5$), a normalization factor calculated based on the geometric mean of the four endogenous control genes was used for each sample.

Mmp20 mRNA was not detected during *E13* (bud stage), *E16* (initial period of bell stage), or postnatal day 1 (secretory root stage). *Mmp20* relative expression increased from the later period of bell stage (6.35 at *E17*) to the secretory crown stage (12.92 at *E19*), when the enamel matrix is secreted. Significant differences between secretory enamel and others stages were observed ($p < 0.05$; Table 5).

4. Discussion

The aetiology of developmental dental alterations is almost certainly heterogeneous, in which genetic and environmental factors contribute to distinct phenotypes. As part of our ongoing effort to understand the molecular mechanism underlying tooth agenesis, we report here a genetic epidemiological approach to identify genetic factors contributing to isolated human tooth agenesis. This is the first report to investigate MMP1, MMP3, and MMP20 in human tooth agenesis. One previous report also in Brazilians did not find association between variation in MMP9 and hypodontia.[20]

Matrix metalloproteinases are a family of proteolytic enzymes that are capable of degrading almost all extracellular matrix proteins. The matrix metalloproteinase family is composed of 23 enzymes that share significant sequence homologies. They can be classified into subfamilies: collagenases, stromelysins, gelatinases, membrane-type matrix metalloproteinases, and others, including a few of the most recently identified.[21] The matrix metalloproteinases and their endogenous inhibitors, the tissue inhibitors of matrix metalloproteinases mediate the maintenance and degradation of the extracellular matrix. It has been demonstrated that matrix

metalloproteinases play a critical role controlling the remodelling of the extracellular matrix during development[11] and matrix metalloproteinases contribute to both normal and pathological tissue remodelling. Physiological roles for matrix metalloproteinases include cell migration, tissue remodelling during organogenesis and growth, wound healing, angiogenesis and tooth formation. Previous studies have suggested matrix metalloproteinases as potential candidate genes for craniofacial alterations based on expression patterns and the roles they play in craniofacial tissues during early embryogenesis.[10,22]

MMP1, is also known as collagenase, is able to initiate breakdown of the interstitial collagens, types I, II, and III. Collagens are the most abundant proteins in the body, which means that MMP1 is important in the remodelling events. During craniofacial development, MMP1 plays a key role in facial and early tooth development. In the bud stage, MMP1 is expressed within both epithelial and mesenchymal cells.[23] Our results provide evidence that variation in MMP1 may contribute to tooth agenesis.

We also investigated a promoter polymorphism in MMP3 (stromelysin-1), but did not find evidence for association with tooth agenesis. MMP3 was chosen for this study because an association between the same MMP3 polymorphism and cleft lip and/or palate was observed.[16] It has been suggested that tooth, lip, and palate development is influenced by the same genes, and evidence for that comes from studies that showed an association between oral clefts and tooth agenesis outside the cleft area. Patients born with oral clefts have a higher risk of presenting tooth agenesis than general population.[24] Recently, MSX1, TGFA, IRF6, and FGFR1,[14,25] all genes that contribute to oral clefts, were associated with tooth agenesis in humans.

MMP20 (enamelysin) is expressed almost 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.[26,27] MMP20 is related to enamel alterations[28,29] and mutations in MMP20have been associated with autosomal recessive forms of amelogenesis imperfecta.[30] Mmp20 knock-out mouse does not process amelogenin properly resulting in altered enamel matrix; the enamel is hypoplastic and delaminates from the dentin.[27] In the developing teeth, MMP20 is expressed primarily during the secretory to late transition stages of amelogenesis and is considered a predominant enzyme for the processing of enamel matrix. MMP20 is present in ameloblasts, odontoblasts, and pulp cells.[26,31]

Although our quantitative real-time polymerase chain reaction suggested Mmp20 expression occurs only during the enamel matrix period, which is in agreement with previous reports of Mmp20 expression only in later stages of dental development,[26,31] the association between a polymorphism in MMP20 and tooth agenesis raises interesting questions about dental development.

It is well established that MMP20 has an important role during enamel development, and our results could reflect the possibility that MMP20 also participate in the remodelling of tooth matrices during the early phases of human tooth organogenesis. Moreover, we may hypothesize that MMP20 participates in the earlier stages of development of only specific dental groups (i.e., in premolars, but not in incisors or molars). Indeed, each tooth group seems to have independent developmental mechanisms and different genetic factors may be involved in the development of each group.[5]

Whilst in our family studies, premolars were the most common affected teeth, molecular studies of odontogenesis in mice focuses in incisor and molar development. Differences in human and mouse dentitions are evident. The tooth formula in mice is reduced in comparison to humans, and includes only one incisor separated by a toothless diastema from the group of 3 molariform teeth. Hence, mice are models that cannot provide insight into premolar development. It has been proposed that the large diastema buds represents vestiges of rodent premolars that were eliminated during mouse evolution, and apoptotic mechanisms are involved.[32,33] Although human premolar agenesis could also be the result of human evolution, one can speculate that discrepancies in human and mouse tooth formula could explain the lack of Mmp20 expression observed in our study in early stages of mouse tooth development, in contrast to the association of MMP20 with human tooth agenesis.

In conclusion, this is the first report to suggest a role for MMP1 and MMP20 in human tooth agenesis. Matrix metalloproteinases are involved in critical processes of early tooth morphogenesis and are viable candidate genes for dental alterations. Differences in the results between the Brazilian and Turkish data sets can be possibly explained by their distinct ethnic origins (as evidenced by different allele frequencies, Table 4). One cannot exclude the possibility of different statistical power between the two data sets. Further investigations should focus on replicating these findings, which will warrant functional studies aiming to define the specific roles of matrix metalloproteinases in the development of dental alterations in humans.

Competing interests

None.

Ethical approval

University of Pittsburgh Institutional Review Board, IRB Number 0511110, Federal University of Rio de Janeiro Ethics Committee, Protocol # 213/04, Istanbul University Ethics Committee, Protocol # 5255.

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