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Association of *MMP3* and *TIMP2* Promoter Polymorphisms with Nonsyndromic Oral Clefts

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Abstract

Background—Oral clefts are common congenital anomalies and result from defects during embryogenesis. The complex etiology is evident by the number of genes and signaling pathways involved in craniofacial development. Matrix metalloproteinases (MMPs) and their inhibitors TIMPs are responsible for tissue remodeling during craniofacial development.

Methods—In this study, we investigated the association of polymorphisms in 14 biologically relevant MMP and TIMP genes in 494 individuals with oral clefts and 413 control individuals from Brazil. Genotypes were generated using Taqman chemistry. Analyses were performed using PLINK software.

Results—Polymorphisms in *MMP3* (rs522616) and *TIMP2* (rs8179096) showed significant association with all cleft types (all clefts, cleft lip/palate, and cleft palate) ($P \leq 0.002$). An additional family-based dataset (881 case-parent trios) from the US was used for confirmation of the association findings ($P < 0.05$). Analysis of gene-gene interaction suggests that *MMP3* and *TIMP2* may interactively contribute to a cleft phenotype.

Conclusions—This study provides new evidence that variation in *MMP3* may contribute to nonsyndromic oral clefts and further supports the involvement of *TIMP2* as a cleft susceptibility gene. Although additional studies are still necessary to unveil the exact mechanism by which *MMP3* and *TIMP2* would contribute to a cleft phenotype, allelic polymorphisms in these genes and their interactions may partly explain the variance of individual susceptibility to oral clefts.

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Keywords

MMP; TIMP; cleft lip; palate; polymorphism; promoter

INTRODUCTION

Nonsyndromic oral clefts account for approximately 65% of all craniofacial anomalies in humans and arise when the fronto-nasal and maxillary prominences of the embryonic face fail to unite. In addition to lifelong medical interventions, significant social and economic complications are imposed to affected individuals and their families (Marazita and Mooney, 2004). The prevalence of oral clefts ranges from 1 in 700 to 2000 births and varies considerably by ancestral origin, with the highest incidence in Asian populations followed by Caucasian and African populations (Mossey and Little, 2002). Oral clefts have a complex etiology and several genes have been suggested to play a role in the susceptibility to oral clefts (Vieira, 2008). By tradition, oral clefts have been referred to as cleft lip with or without cleft palate (CL/P) and cleft palate only (CPO), due to differences in embryology (Murray, 2002).

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases responsible for tissue remodeling due to their role in extracellular matrix remodeling, degrading molecules from the extracellular matrix and basement membranes, and in promoting cell migration and proliferation. Together with their tissue inhibitors (TIMPs), MMPs have key roles during embryogenesis and homeostasis of adult tissues (Nagase et al., 2006). Remodeling of the extracellular matrix is a crucial event to accommodate specific interactions that ultimately result in proper tissue morphogenesis during craniofacial and palate development. Hence, disturbances that affect extracellular matrix remodeling may in turn lead to defects during palatogenesis (Iamaroon et al., 1996).

Expression of MMPs and TIMPs mRNA and protein has been detected in the developing mouse palate, in specific spatial and temporal distribution patterns in areas where their preferred substrates are located (Morris-Wiman et al., 2000; Blavier et al., 2001; Brown et al., 2002; de Oliveira Demarchi et al, 2010). Moreover, absence of MMP activity has been shown to result in failure of palatal shelf fusion *in vitro* and *in vivo* (Blavier et al., 2001; Brown et al., 2002). Although a single MMP gene knockout in mouse does not cause clefting, inactivating mutations of both *Mmp14* and *Mmp16* yielded severe structural and craniofacial defects including a severe dysfunction in palatal shelf formation leading to cleft palate in 80% of the mouse embryos (Shi et al., 2008).

We have previously reported the association of a polymorphism in the *MMP3* gene (rs3025058) with cleft lip/palate in a population-based sample from Brazil (Letra et al., 2007). This polymorphism consists of a common adenine insertion/deletion polymorphism at position -1171 of the *MMP3* gene promoter, where the 5A allele has been correlated with higher transcriptional activity (Ye et al., 1996). Variants in or nearby *MMP25* and *TIMP2* have also presented suggestive association with oral clefts (Blanton et al., 2004; Nikopensius et al., 2011).

Taken together, previous evidence from biological and human studies support a role for MMP and TIMP genes as likely candidate genes for the occurrence of oral clefts. In the present study, we investigated 14 biologically relevant MMP and TIMP genes for association with cleft phenotypes in a case-control dataset from Brazil. An additional family-based dataset from the US was used for confirmation of the association findings.

MATERIALS AND METHODS

Sample Population

This population has been described in part elsewhere (Letra et al., 2007). The case-control dataset consisted in 907 unrelated individuals recruited at the Hospital of Rehabilitation and Craniofacial Anomalies, Bauru Dental School, University of Sao Paulo, and at the Center for Treatment of Craniofacial Anomalies, Rio de Janeiro, Brazil. Of these, 494 individuals (aged 1–59, mean age 18) considered nonsyndromic according to geneticists' assessments, presented an oral cleft: 10 with cleft lip (CL), 411 with cleft lip and palate (CLP), and 73 with cleft palate (CP). The controls were comprised of 413 unrelated individuals (aged 5–72, mean age 28) without clefts or family history of clefts. To control for possible population admixture effects in cases and controls, only individuals from the Southeastern region of Brazil, reporting Caucasian ascendants to the second generation were included in the study.

This study was approved by the Institutional Review Boards at each subject recruitment site, at the University of Pittsburgh, and the University of Texas Health Science Center at Houston. Participants signed an informed consent and provided a saliva sample as source of genomic DNA.

Selection of Candidate Genes and Single Nucleotide Polymorphisms (SNPs)

We selected ten MMP and four TIMP genes to be studied considering: 1) expression during craniofacial development; 2) location being in or near a cleft locus; 3) presence of substrate in the palatal matrix; 4) previous association with oral clefts. We excluded genes whose roles are not relevant for craniofacial development, or when available information was not sufficient to fit the selection criteria. Next, we selected 45 polymorphisms based on their likelihood to have functional consequences (i.e., located in the promoters, exons, or near exon/intron boundaries), or if considered tag-SNPs as surrogates for the linkage disequilibrium blocks surrounding the candidate gene. We used information from the NCBI dbSNP (http://www.ncbi.nlm.gov/SNP/) and the International HapMap Project (http://www.hapmap.org) databases (Table 1).

Genotyping

Genomic DNA was extracted from saliva as described elsewhere (Trevillato and Line, 2000). DNA concentration was estimated by measuring the absorbance at 260nm in a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). Genotypes were generated using Taqman chemistry (Ranade et al., 2001)in an ABI 7900 instrument (Applied Biosystems, Foster City, CA). Reactions were carried out with the use of standard conditions as suggested by the manufacturer. For quality control purposes, negative control reactions were performed using no nucleic acid template; positive control reactions were performed using samples of known genotypes. Genotyping was performed blind to sample status.

Statistical Analyses

Markers were assessed for Hardy-Weinberg equilibrium in controls and affected individuals using a Pearson's chi-square test. LD measures were calculated, and haplotype blocks were defined using the confidence interval method. Haplotype frequencies were estimated using Haploview 4.1 software (http://www.broadinstitute.org/haploview).

Differences in allele frequencies between individuals with oral clefts and controls were compared for each SNP using PLINK software version 1.05 (Purcell et al., 2007).Due to the small number of individuals in the CL group (n=11), these individuals were grouped with the CLP individuals, thus forming a cleft lip with or without cleft palate (CL/P) analysis

group. Analyses were performed comparing all clefts with controls, and then comparing CL/P, and CP with controls. Allelic odds ratios (ORs) and 95% confidence intervals (CI) were also calculated. We corrected for multiple testing using the Bonferroni method considering the number of variables and tests performed, and P-values below 0.002 were considered significant. Logistic regression analyses were performed under an additive model to assess the significance of the minor allele copy number on the association. Haplotype analyses were performed using 2-, 3-, and 4-SNP sliding windows.

Prediction of SNP functionality

We performed *in silico* analyses of SNP function to search for potential transcription factor binding sites in the sequence of the associated variants. We used the following prediction programs: AliBaba2.1 (Grabe, 2002), TESS (Transcription Element Search System) (Schug, 2008) and FastSNP (Yuan et al., 2006).

Confirmation Dataset

We tested 5 SNPs of interest in *MMP3* and 7 SNPs in *TIMP2* in an independent dataset of 881 CL/P families ascertained in the US (545 non-Hispanic white and 336 Hispanic families from Texas). These included the SNPs associated in the case-control dataset. Details are available as Supplemental Material.

Analyses were performed stratified by population using Pedigree Disequilibrium test (PDT), Genotype-pedigree disequilibrium test (Geno-PDT), and Association in the Presence of Linkage (APL) as previously described (Chiquet et al., 2007). A $P \leq 0.05$ was considered statistically significant.

Gene-Gene Interaction

We looked for evidence of gene-gene interaction between *MMP3* and *TIMP2* by calculating the proportion of cleft cases presenting at least one copy of the associated alleles (A and C, respectively), and comparing to controls. Analysis was performed using Mantel-Haenszel statistics. A P-value ≤ 0.05 indicated statistical significance.

RESULTS

Eleven SNPs were not informative in our population (Table 2). Minor allele frequency was < 10% for *MMP14* rs17123036, *MMP27* rs12099177, and *TIMP1* rs1062849. *TIMP2* rs4789940 showed deviation from Hardy-Weinberg equilibrium. These markers were excluded from further analyses.

Association analyses

The results of the association analyses are presented in Table 2. We found significant differences in allelic frequencies for SNPs in the promoter of *MMP3* and *TIMP2* genes between all cleft phenotypes and controls. The ancestral allele in *MMP3* rs522616 was associated with increased risk of oral clefts (P=0.000003 for all clefts; P=0.0003 for cleft lip/palate; and P=0.002 for cleft palate). Similarly, the ancestral allele in *TIMP2* rs8179096 also showed significant association with all clefts (P=0.0004), P=0.002 for cleft lip/palate; and P=0.0007 for cleft palate) (Table 2). Under a nominal threshold of 0.05, SNPs in *MMP9, MMP16* and *TIMP1* also showed association with cleft phenotypes. A nonsignificant trend for association of *MMP3* rs3025058 (associated in the original report by Letra et al., 2007) was also observed in the CP group (p=0.08) (Table 2). Logistic regression supported the individual associations for *MMP3* rs522616 (P=0.00002 for all clefts, P=0.0009 for cleft lip and palate, and P=0.006 for cleft palate) and *TIMP2* rs8179096 (P=0.004 for all clefts; P=0.01 for cleft lip and palate, and P=0.02 for cleft palate) (data not shown).

Haplotype analyses revealed the association of neighboring SNPs in *MMP3* with all cleft types. Of note, haplotypes including SNPs rs522616 and rs3025058 were strongly associated with CL/P and CP phenotypes (P≤0.002) (Table 3).

MMP3-TIMP2 Interaction

Analysis of the *MMP3* and *TIMP2* genotypes in the case-control dataset showed association and suggestive evidence of genetic interaction in cleft individuals carrying at least one copy of the ancestral allele A from *MMP3* rs5622616 and one or 2 copies of the ancestral allele C from *TIMP2* rs8179096 (P=0.000001) (Supplementary Table 1).

Confirmation of association findings

Considering the individual SNP analysis for *MMP3*, the best association results were seen with rs522616 in Hispanic families with CL/P without family history of clefts (P=0.019) whereas two additional *MMP3* SNPs, rs639752 and rs520540, showed association with CL/P in Caucasian families with positive cleft history (P=0.04, for both) (Supplementary Table 3). For *TIMP2*, the best findings were seen with SNP rs8179096 in Caucasian (p=0.03) and Hispanic (p=0.01) families; SNPs rs6501254 and rs9894295, also showed association values (p=0.04 and p=0.03, respectively) (Supplementary Table 4). In the families without history of clefts, we found association of rs8179096 and rs6501257 (p=0.01 and p<0.03, respectively) in Hispanics, whereas SNPs r6501254, rs9894295, rs4789860 and rs6501265 showed nominal association in Caucasians (0.02≤ p ≤0.05). In the presence of family history of clefts, the best results were seen with SNPs rs7212662 (p=0.01) and rs6501254 (p=0.02) in Caucasians, and SNP rs9894295 (p=0.03) in Hispanics (Supplementary Table 4).

Prediction of SNP functionality

In silico analysis of SNP function suggests that the associated allele A (ancestral) in *MMP3* rs522616 creates a binding site for transcription factor *CDX1* (caudal type homeobox 1), that is not present with the minor allele G, whereas allele G creates a binding site for transcription factor *GR* (glucocorticoid receptor) that is not present with the ancestral allele A. Both C and T alleles in *TIMP2* rs8179096 are predicted to harbor binding sites for *Sp1* and Nuclear Factor-kappaB (NFkB) (data not shown).

DISCUSSION

Considerable biological evidence implicates MMPs as key molecules during palatogenesis for their role in extracellular matrix remodeling (Morris-Wiman et al., 2000; Blavier et al., 2001; Brown et al., 2002; Brown and Nazarali, 2010; de Oliveira Demarchi et al, 2010). In this study, we investigated 45 polymorphisms in 14 MMP and TIMP genes relevant for craniofacial development as candidates for oral clefts in a large case-control dataset from Brazil. We found strong association of *MMP3* and *TIMP2* with all clefts, cleft lip/palate, and cleft palate phenotypes. Of note, while the association of *MMP3* was stronger in the CL/P group, the association with *TIMP2* appears more significant in the CP group. The associated variants, *MMP3* (rs522616) and *TIMP2* (rs8179096), are both located in the gene promoters and may have regulatory effects on gene transcription and function, although their exact functions have not yet been elucidated. Haplotypes containing the individually associated *MMP3* rs522616 were also strongly associated with oral clefts, particularly with CL/P. We included an additional large family-based dataset from the US for confirmation of the association findings and observed the association of both *MMP3* rs522616 and *TIMP2* rs8179096 with CL/P in families of Caucasian and Hispanic descent, with and without family history of clefts. Additional SNPs also showed association with CL/P in these families. While *MMP3* rs522616 showed association with CL/P in individuals of Hispanic background and no family history of clefts, two additional SNPs, rs520540 and rs639752,

showed association in individuals of Caucasian background and positive family history of clefts. Similarly, the association of *TIMP2* rs8179096 appears stronger in Hispanics with no family history of clefts. These findings suggest that variations in these genes may play a role in the susceptibility to oral clefts, as verified by the associations in Brazilian and US populations.

The enzyme MMP-3, also known as stromelysin-1, degrades a wide range of substrates, including type III, IV and V collagens, proteoglycans, fibronectin and laminin, all of which are abundantly present in the palatal matrix (Nagase et al., 2006). MMP-3 is also capable of activating other MMPs, making it likely a key player in ECM degradation and remodeling (Ye et al., 1996). During the process of palatogenesis in mice, expression of Mmp-3 has been detected *in vivo* subjacent to the medial edge epithelia following contact of the palatal shelves(Blavier et al., 2001) and at higher levels in fused palates than in palates that failed to fuse (Brown et al., 2002). Moreover, induction of Mmp-3 expression has been shown to result in cleavage of cadherin, loss of the epithelial phenotype and subsequent stable conversion of epithelia into mesenchyme (Lochter et al., 1997), a mechanism suggested to occur at the final stages of palatogenesis. Taken together, these findings imply that Mmp-3 induces epithelial breakdown at the medial epithelial edge of fusing palatal shelves if present in adequate amounts, and that insufficient or null expression may lead to failures in palatal fusion. Corroborating with these observations, a recent transcriptome analysis comparing dental pulp stem cells from cleft lip/palate patients and controls revealed 87 differentially expressed genes and downregulation of *MMP3* in cleft patients when compared to controls (Bueno et al., 2011).

We have previously reported the association of a polymorphism in *MMP3* (rs3025058) with nonsyndromic oral clefts in a smaller sample population (Letra et al., 2007). This polymorphism consists of a common adenine insertion/deletion polymorphism (5A/6A) at position -1171 of the *MMP3* promoter region and modulates transcription and local expression of the MMP-3 protein. It has been demonstrated that the 6A allele expresses a roughly 2-fold lower amount of gene product as compared with the 5A allele (Ye et al., 1996). Additional studies have revealed binding of putative transcription factors to this region. The difference in promoter activity is probably due to the differential binding of the transcriptional repressor to the 6A allele (Mercapide et al., 2003). In the present study, we only observed a nonsignificant trend for the individual association of SNP rs3025058 and cleft palate, whereas haplotypes containing rs3025058 and rs522616 alleles were strongly associated with CL/P and CP phenotypes. Discrepancies between our previous and the present study include a larger sample size and investigation of numerous additional polymorphisms, which make direct comparisons rather difficult. Similarly to SNP rs3025058 though, the *MMP3* SNP r522616 associated in the present study is also located in the gene promoter, at -709bp from the transcription start site, although no evidence is available on allele-specific functions. Functional polymorphisms derived from nucleotide substitutions have been reported to regulate MMP expression by altering the interaction between cis-elements in the promoter and transcription factors regarded as important for embryonic development (Ye, 2000).Our *in silico* analysis of SNP function did not predict any damaging effects for the associated *MMP3* -709A/G variant although predicted binding sites for putative transcription factors were different for each allele. The presence of the ancestral allele A, associated with oral clefts in this study, is predicted to harbor a binding site for transcription factor *CDX1* (caudal type homeobox 1). *CDX1* is a member of the caudal-type homeobox family of genes required for anterior-posterior axial skeletal identity in mammals. During murine embryonic development, *Cdx1* is expressed along the embryonic axis from day 7.5 postcoitum until day 12, and inactivation of this gene resulted in abnormalities in craniofacial and vertebral formation, however palatal bone development was not examined (Subramanian et al., 1995). Moreover, in the course of positional cloning

of the gene involved in the pathogenesis of Treacher Collins syndrome (OMIM 154500), a disorder of craniofacial development characterized by coloboma of the lid, micrognathia, microtia, and cleft palate, it was determined that the *CDX1* locus is situated within a region of approximately 900 kb proximal to the *TCOF1* gene, etiologic for the syndrome. While these observations do not provide evidence for a direct relationship between *MMP3, CDX1* and an increased risk of oral clefts, additional studies including sequencing of conserved and putative regulatory elements, followed by functional validation might provide insights into the potential role of *MMP3* and downstream effectors in the pathogenesis of oral clefts.

A promoter variant in *TIMP2* (rs8179096, -180C/T) was also strongly associated with oral clefts in the case-control dataset whereas several additional variants showed association in the family-based dataset. *TIMP2* has been previously associated with nonsyndromic CL/P in Northeastern European populations, although the associated variants reflect intronic regions of unknown function (Nikopensius et al., 2011). These observations suggest that the associated markers may be in linkage disequilibrium with the true causal variant and indirectly contribute to a cleft phenotype. TIMPs are inhibitors of MMPs. In the extracellular matrix, they form non-covalent 1:1 stoichiometric complexes with MMPs and inhibit MMP activation or function and thereby restrict extracellular matrix breakdown (Woessner, 1999). During palatogenesis, Mmp and Timp expression is highly regulated to control tissue remodeling, and an imbalance in favor of *Timp2* has been shown to increase the risk of a cleft (Blavier et al., 2001).

Several genes are likely to be jointly involved in the etiology of oral clefts (Schliekelman and Slatkin, 2002), and numerous gene variants have been individually implicated in increasing an individual's susceptibility to develop an oral cleft. In general, the associated variant will show the minor allele frequency lower in the cases than in the controls, thus implying the functionality of the variant. In our study, the individual associations with *MMP3* and *TIMP2* were both with the ancestral alleles, which could suggest that the associated variants may be surrogates for true etiologic variants in the same linkage disequilibrium block. Further, our interaction analysis shows that the presence of one or two copies of the ancestral alleles for the respective *MMP3* and *TIMP2* variants is more frequently observed in cleft individuals. *MMP3* and *TIMP2* are located on different chromosomes (11q and 17q respectively), therefore interaction due to linkage disequilibrium is not expected. Rather, the interaction may reflect the overtransmission of these alleles more frequently in cleft individuals. *TIMP2* has the ability to inhibit *MMP3* (Woessner, 1999), and the observed allelic combinations may exert effects on *MMP3* activity with consequences during palatogenesis. To date, significant association between SNPs in and around *IRF6* (interferon regulatory factor-6) and CL/P was previously shown in multiple populations and independently replicated (Dixon et al., 2011). Similar to our results, the first associated SNP (rs2235371) that changes valine to isoleucine at amino acid position 274 (V274I) was found to be significantly associated with CL/P in Asian and Amerindian populations, and the associated G allele was the ancestral allele, with a very high (97%) frequency in European and African populations (Zucchero et al., 2004). This V274I mutation was later shown to be in linkage disequilibrium with an *IRF6* enhancer variant (rs642961), which in turn has been implicated as the causal variant in CL/P (Rahimov et al., 2008).

In summary, this study provides new evidence that variation in *MMP3* may contribute to nonsyndromic oral clefts and further supports the involvement of *TIMP2* as a cleft susceptibility gene. Although additional studies are still necessary to unveil the exact mechanism by which *MMP3* and *TIMP2* would contribute to a cleft phenotype, allelic polymorphisms in these genes and their interactions may partly explain the variance of individual susceptibility to oral clefts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Details of the genes and single nucleotide polymorphisms investigated.

Gene	dbSNP ID	Location (chromosome, base position)	SNP Function	Position in sequence (nucleotide change)[a]	Alleles[b]
MMP2	rs243865	chr.16, 54069297	5′ near gene - promoter	-1306 C/T	C/T
	rs2285053	chr.16, 54069878	5′ near gene - promoter	-735 C/T	C/T
	rs243847	chr.16, 54081499	Intron		T/C
	rs2287074	chr.16, 54084614	Coding synonymous	Thr460Thr (exon 9)	G/A
	rs9923304	chr.16, 54087802	Intron		C/T
	rs28730814	chr.16, 54088365	Intron		A/G
	rs11639960	chr.16, 54090771	Intron		A/G
MMP3	rs639752	chr.11, 102212549	Intron		T/G
	rs650108	chr.11, 102213997	Intron		G/A
	rs520540	chr.11, 102214635	Coding synonymous	Ala362Ala (exon 8)	G/A
	rs3025065	chr.11, 102216192	Coding synonymous	Gly264Gly	A/G
	rs11606831	chr.11, 102218625	Missense	His113Pro	G/T
	rs679620	chr.11, 102218830	Missense	Lys45Glu (exon 2)	G/A
	rs522616	chr.11, 102220258	5′ near gene – promoter	-709 A/G	A/G
	rs3025058	chr.11, 102221152	5′ near gene - promoter	-1171 5A/6A	5A/6A
MMP7	rs11568819	chr.11, 101906843	5′ near gene – promoter	-153 C/T	C/T
	rs11568818	chr.11, 101906871	5′ near gene – promoter	-181 A/G	A/G
MMP9	rs3918253	chr.20, 44072918	Intron	49 5′ exon 4	C/T
	rs17576	chr.20, 44073632	Missense	Gln279Arg	A/G
	rs17577	chr.20, 44076518	Missense	Gln668Arg (exon 12)	G/A
MMP10	rs17860973	chr.11, 102152308	Missense	Gly282Glu	G/A
	rs17860971	chr.11, 102152662	Missense	Phe226Leu	T/G
	rs17293607	chr.11, 102155599	Missense	Gly65Arg (exon 2)	C/T
MMP13	rs478927	chr.11, 102330036	Intron		A/G
	rs12295719	chr.11, 102331288	Missense	Asp89HIs	C/G
	rs2252070	chr.11, 102331749	5′ near gene – promoter	-77 A/G	G/A

Gene	dbSNP ID	Location (chromosome, base position)	SNP Function	Position in sequence (nucleotide change)[a]	Alleles[b]
MMP14	rs17123036	chr.14, 22375133	5′ near gene – promoter	-165 GT	A/G
	rs1042704	chr.14, 22382434	Missense	Asn273Asp	A/G
MMP16	rs2616490	chr.8, 89150846	Missense	Ile415Val	A/G
	rs7828497	chr.8, 89408837	5′ near gene – promoter		T/G
MMP25	rs2360167	chr.16, 3036160	5′ near gene - promoter		G/T
MMP27	rs61995943	chr.11, 102067834	Nonsense	Ser472OPA	C/G
	rs2509010	chr.11, 102067910	Missense	Asp447Asn	C/T
	rs12099177	chr.11, 102081592	Missense	Arg22Trp	G/A
	rs11225389	chr.11, 102081678	5′ untranslated region		C/A
TIMP1	rs6520279	chr.X, 47204350	Intron		C/T
	rs5906435	chr.X, 47204664	Intron		C/T
	rs1062849	chr.X, 47330943	Missense	Ser178Phe	C/T
TIMP2	rs4789940	chr.17, 74389266	Intron		G/T
	rs8179096	chr.17, 74433244	5′ near gene – promoter	-177 C/T	C/T
	rs9747145	chr.17, 74434703	5′ near gene – promoter		G/T
	rs9894526	chr.17, 74438681	5′ near gene - promoter		C/T
TIMP3	rs34586282	chr.22, 31526804	Frameshift	Cys155V	-/G
	rs34334473	chr.22, 31585191	5′ untranslated region		A/G
TIMP4	rs3755724	chr.3, 12175906	5′ near gene promoter	-55C/T	C/T

[a] According to NCBI Reference Assembly.

[b] Ancestral allele listed first according to NCBI Reference Assembly.

Table 2

Results of association tests with MMP and TIMP gene polymorphisms and nonsyndromic oral clefts.

SNP	Minor Allele	Minor allele frequency		All Clefts (n=494)			Cleft Lip/Palate (n=421)			Cleft Palate (n=73)		
		Cases (n=494)	Controls (n=413)	P-value	Odds ratio	95% CI	P-value	Odds ratio	95% CI	P-value	Odds ratio	95% CI
MMP2												
rs243865	T	0.17	0.18	0.7534	0.96	0.75–1.23	0.9349	1.01	0.78–1.31	0.1115	0.66	0.40–1.10
rs2285053	T	0.13	0.15	0.2534	0.85	0.65–1.12	0.2577	0.85	0.63–1.13	0.4920	0.83	0.50–1.40
rs243847	C	0.36	0.37	0.8483	0.98	0.81–1.20	0.8652	1.02	0.83–1.25	0.3408	0.84	0.58–1.21
rs2287074	A	0.38	0.39	0.6948	0.96	0.79–1.16	0.7184	0.96	0.79–1.18	0.8303	0.96	0.67–1.37
rs9923304	T	0.37	0.37	0.8485	0.98	0.81–1.19	0.8040	0.97	0.79–1.20	0.9465	0.99	0.69–1.42
rs11639960	G	0.32	0.32	0.8882	0.99	0.81–1.20	0.9595	1.01	0.81–1.24	0.6831	0.92	0.63–1.35
MMP3												
rs639752	G	0.48	0.46	0.3896	1.09	0.90–1.31	0.9479	0.99	0.81–1.21	*0.0102*	1.58	1.11–2.25
rs650108	G	0.32	0.33	0.7042	0.96	0.79–1.17	0.7256	1.04	0.84–1.28	*0.0448*	0.67	0.45–0.99
rs520540	A	0.45	0.46	0.6774	0.96	0.80–1.16	0.2865	0.90	0.74–1.10	0.0924	1.35	0.95–1.91
rs679620	A	0.45	0.44	0.7039	1.04	0.86–1.25	0.7826	0.97	0.79–1.19	0.0519	1.41	1.00–2.00
rs522616	G	0.28	0.39	**0.000003**	0.62	0.51–0.76	**0.0003**	0.68	0.55–0.84	0.0022	0.55	0.37–0.81
rs3025058	5A	0.41	0.41	0.9395	0.99	0.82–1.20	0.5099	0.93	0.76–1.14	0.0821	1.36	0.96–1.93
MMP7												
rs11568818	G	0.42	0.42	0.7878	1.03	0.85–1.24	0.6497	1.05	0.86–1.28	0.7246	0.94	0.66–1.34
MMP9												
rs3918253	C	0.51	0.47	0.1493	1.15	0.95–1.38	0.0266	0.80	0.66–0.97	0.2550	0.82	0.57–1.16
rs17576	G	0.31	0.32	0.7042	0.96	0.78–1.17	0.8695	0.98	0.79–1.22	0.6280	0.91	0.62–1.33
rs17577	G	0.11	0.12	0.8298	0.97	0.72–1.29	0.6063	0.92	0.68–1.26	0.8139	1.07	0.63–1.80
MMP10												
rs17293607	T	0.15	0.18	0.0602	0.78	0.60–1.01	0.0711	0.78	0.59–1.02	0.6971	0.91	0.57–1.45
MMP13												
rs478927	G	0.37	0.34	0.1400	1.19	0.94–1.48	0.0905	1.23	0.97–1.57	0.3157	0.78	0.49–1.26
rs2252070	G	0.34	0.33	0.7859	1.03	0.84–1.25	0.6330	1.05	0.85–1.29	0.1854	0.77	0.53–1.13

SNP	Minor Allele	Minor allele frequency		All Clefts (n=494)			Cleft Lip/Palate (n=421)			Cleft Palate (n=73)		
		Cases (n=494)	Controls (n=413)	P-value	Odds ratio	95% CI	P-value	Odds ratio	95% CI	P-value	Odds ratio	95% CI
MMP14												
rs1042704	A	0.14	0.13	0.7171	1.05	0.80–1.38	0.9188	0.98	0.73–1.32	0.0551	1.55	0.99–2.44
MMP16												
rs7828497	G	0.32	0.26	*0.0125*	1.30	1.05–1.60	*0.0182*	1.30	1.05–1.61	0.3107	1.22	0.83–1.78
rs2616490	G	0.39	0.37	0.3350	1.10	0.90–1.32	0.1529	1.16	0.95–1.42	0.2491	0.81	0.56–1.16
MMP25												
rs2360167	T	0.24	0.22	0.6027	1.06	0.85–1.32	0.7500	1.04	0.82–1.32	0.4212	1.18	0.79–1.77
MMP27												
rs2509010	T	0.23	0.23	0.9885	1.00	0.80–1.25	0.9578	1.01	0.80–1.27	0.7531	1.07	0.71–1.61
rs11225389	A	0.17	0.19	0.1915	0.85	0.67–1.08	0.0977	0.80	0.62–1.04	0.1533	1.35	0.89–2.03
TIMP1												
rs5906435	T	0.28	0.25	0.2301	1.16	0.91–1.48	0.7173	1.05	0.81–1.36	*0.0446*	1.54	1.01–2.36
rs6520279	C	0.43	0.42	0.5119	1.16	0.91–1.48	0.8497	1.02	0.81–1.29	0.4273	1.17	0.79–1.74
TIMP2												
rs8179096	T	0.08	0.13	**0.0004**	0.57	0.41–0.77	**0.0018**	0.59	0.42–0.82	**0.0007**	0.23	0.09–0.58
rs9894526	T	0.47	0.45	0.3055	1.10	0.91–1.33	0.2676	1.12	0.92–1.36	0.9223	0.98	0.69–1.40

*P≤ 0.002 indicates statistical significance under Bonferroni correction (in bold).

The following SNPs were non informative or presented minor allele frequency < 10% in the studied population and therefore excluded from the association analyses: rs28730814 (*MMP2*), rs3025065 and rs11606831 (*MMP3*), rs11568818 (*MMP7*), rs17860973 and rs17860971 (*MMP10*), rs12295719 (*MMP13*), rs17123036 (*MMP14*), rs61995943 and rs12099177 (*MMP27*), rs9747145 (*TIMP2*), rs34586282 and rs34334473 (*TIMP3*), and rs3755724 (*TIMP4*). *TIMP2* rs4789940 showed deviation from Hardy-Weinberg equilibrium and was also excluded from further analyses.

Table 3

Results of haplotype analysis for *MMP3* variants in all clefts, cleft lip/palate and cleft palate groups.

	Window	rs639752	rs650108	rs520540	rs679620	rs522616	rs3025058
All Clefts	1 SNP	0.39	0.70	0.67	0.70	**0.000003 (A)**	0.93
	2 SNP	0.62					
			0.009 (GA)				
				0.03 (AG)			
					0.86		
						0.73	
	3 SNP	**0.005 (TAA)**					
			0.007 (AAG)				
				0.64			
					0.79		
	4 SNP	**0.004 (TAAG)**					
			0.006 (AAGA)				
				0.28