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Analysis of MMP-9 and TIMP-2 Gene Promoter Polymorphisms in Individuals with Hypodontia

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Hypodontia, the congenital absence of one or a few teeth, is one of the most common developmental alterations of human dentition. It may cause masticatory and speech dysfunctions and create esthetic problems with orthodontic and prosthetic implications. MMP-9 is an important member of the matrix metalloproteinase (MMP) family that participates in remodeling of extracellular matrix during tooth development. A C-to-T base exchange at position -1562 creates two different alleles, and the CT and TT genotypes promote high activity of the MMP-9 gene promoter. Tissue inhibitor of metalloproteinase-2 (TIMP-2) regulates the activity of MMPs in the extracellular matrix and is co-expressed with gelatinases A (MMP-2) and B (MMP-9) during mouse tooth morphogenesis. A polymorphism in the TIMP-2 gene promoter at position -418 has been found in a Sp-1 binding site. In this study, the association between these DNA polymorphisms and hypodontia has been investigated. The significance of differences in frequencies of polymorphisms in control and test groups was assessed by Chi-square test ($p<0.05$). Data suggested that MMP-9 gene promoter polymorphism was not associated with hypodontia. The high frequency of GG genotype in the TIMP-2 gene promoter showed that this site was unsuitable for studies of DNA polymorphism-disease associations in the studied population.

Key Words: MMP-9, TIMP-2, hypodontia, tooth agenesis.

INTRODUCTION

Hypodontia, the congenital absence of one or a few teeth, is one of the most common developmental alterations of human dentition. Although it is not a serious public health problem, hypodontia may cause masticatory and speech dysfunctions and create esthetic problems with orthodontic and prosthetic implications. Over the past few years, the importance of several genes in tooth development has been shown by the lack of teeth in mutant knockout mice models (1). Autosomal dominant forms of tooth agenesis are due to mutations in human MSX1, PAX9 and AXIN2 genes (2,3). However, the origin of hypodontia, the most common form of tooth agenesis in humans, remains uncertain. Although the exact molecular mechanisms involved in tissue interactions that regulate tooth development are unknown, there is evidence that extracelullar matrix (ECM) molecules may play a major role (4).

In addition to provide a scaffold for cell adhesion and migration, the ECM also has an important role on the modulation of cell behavior (5). ECM molecules can recruit and store signaling molecules. Moreover, ECM components can act as signaling molecules themselves. The turnover of ECM is mainly regulated by a class of proteolytic enzymes known as matrix metalloproteinases (MMPs). MMPs constitute an important family of zinc-dependent endopeptidases, which are able to degrade most, if not all, components of extracellular matrix (6). The activity of metalloproteinases on ECM is regulated by specific MMPs inhibitors, known as tissue metalloproteinases inhibitors (TIMPs). The TIMP family consists of four members: TIMP-1, -2, -3 and -4. These molecules inhibit the proteolytic activity of activated MMPs by forming a 1:1 stoichiometric inhibitory complex with the enzyme. TIMP-2 is coexpressesed with gelatinases A (MMP-2) and B (MMP-9) during mouse tooth morphogenesis. Disturbances in MMP-TIMP interactions have been implicated in the etiology of some diseases, such as arthritis, pulmonary emphysema, atherosclerosis and periodontitis, in which the loss of ECM is a major feature (7).

MMP-9 and TIMP-2 participate in the remodeling of tooth-specific matrices that accompanies the developmental events as these molecules are expressed during early tooth development (8,9). However, there

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are no studies correlating disturbances in MMP9 or TIMP2 expression in tooth development. MMP-9 gene presents a functional C-to-T single nucleotide polymorphism (SNP) at position -1562, which affects transcription. The CC genotype has been shown to decrease transcriptional activity (10). This MMP-9 polymorphism has been associated with high risk for vascular diseases, such as coronary atherosclerosis (10). A polymorphism in the TIMP-2 gene promoter at position -418 has been described and corresponds to a G to C substitution in a consensus sequence for the Sp1 binding site (11). Polymorphisms in the promoter region of TIMP-2 gene have been associated with chronic obstructive pulmonary disease (11). Because such polymorphisms alter the expression of MMP-9 and TIMP-2 genes, the analysis of their distribution in populations bearing tooth agenesis may provide a direct evidence of the importance of these genes on the development of dentition in humans. In this study, the association between these DNA polymorphisms and hypodontia has been investigated.

MATERIAL AND METHODS

Fifty-two unrelated Caucasian individuals with hypodontia (Fig. 1) and 48 healthy Caucasian control individuals (without hypodontia) were interviewed and documented. Congenital absence of teeth was confirmed radiographically. No other dental anomalies were observed. For sampling of epithelial buccal cells, the volunteers undertook 5 mL of 3% glucose mouthwash and the oral mucosa was scraped with a sterile wooden spatula. The tip of the spatula was then shacked into the retained mouthwash solution. Oral epithelial cells were pelleted by centrifugation at 2000 rpm for 10 min. The supernatant was discarded and the cell pellet was resuspended in 500 µL extraction buffer [10 mM Tris-HCl (pH 7.8), 5 mM EDTA, 0.5% SDS]. The samples were then frozen at -20°C until use for DNA extraction. After defrost, samples were incubated overnight with 100 ng/mL proteinase K (Sigma Chemical Co., St. Louis, MO, USA) at 37°C with agitation. DNA was then purified by sequential phenol/chloroform extraction and salt/ethanol precipitation. DNA was dissolved in 70 µL TE buffer [10 mM Tris (pH 7.8), 1 mM EDTA]. The concentration was estimated by measurements of OD_{260}.

Thereafter, the sequence from -1809 to -1374 in MMP-9 gene promoter was amplified by polymerase chain reaction (PCR) with primers 5'-GCCTGGCACATAGTAGGCCC-3' (forward) and 5'-CTTCCTAGCCAGCCGGCATC -3' (reverse). PCR was done in a total volume of 50 µL, containing 500 ng genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 1 µM of each primer, 200 µM each dATP, dCTP, dGTP and dTTP, and 2.5 units *Taq* DNA polymerase (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The solution was incubated for 3 min at 95°C, followed by 35 cycles of 1 min at 95°C, 45 s at 65°C and 45 s at 72°C, with a 7-min final extension at 72°C.

A fragment of 176 bp of the TIMP-2 gene promoter was PCR amplified with primers 5'-GGATCCTGTCAGTTTCTCAA-3' (forward); 5'-TTTCCCCTTAGCTCGACTCT-3' (reverse). PCR was carried out in a total volume of 50 µL, containing 500 ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 1 µM of each primer, 200 µM of each dATP, dCTP, dGTP and dTTP, and 4 units of Taq DNA polymerase (Amersham Pharmacia Biotech AB). The solution was incubated for 3 min at 95°C, followed by 35 cycles of 1min at 95°C, 1 min at 55°C, and 1 min at 72°C, with a final extension of 72°C for 7 min.

A 4-µL aliquot of MMP-9 PCR products was mixed with a 8 µL solution containing 1 µL 10x NE Buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9), 0.3 µL *PaeI* (20,000 units/mL) (New England Biolabs, Inc., Beverly, MA, USA) and 6.7 µL sterile deionized H_2O. The solution was incubated at 37°C for 16 h. For analysis of TIMP-2 polymorphism, a 3-µL aliquot of TIMP-2 PCR products was mixed with a 17 µL solution containing 2 µL 10x NE Buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9), 0.5 µL *HgaI* (2,000 U/mL) (New England Biolabs, Inc.) and 14.5 µL sterile deionized H_2O. The solution was incubated at 37°C for 16 h. The digest was mixed 5 µL of loading buffer and electrophoresed on a 10% vertical polyacrylamide gel. The DNA bands were evidenced by the rapid silver staining method (12).

The significance of differences in frequencies of polymorphisms in both groups was assessed by Chi-squared test (x^2). Clump software was used to assess differences between control and test groups. This program is designed for use in genetic case-control studies where multiple alleles are being considered and the observed frequencies of some alleles are rare (13). Statistically significant differences were set at $p<0.05$.

C-1562T	Individual	8R	7R	6R	5R	4R	3R	2R	1R	1L	2L	3L	4L	5L	6L	7L	8L
CC	1	*															*
		*															*
CC	2				*												*
CC	3																
		*															*
CC	4																*
		*															*
CC	5																
		*															*
CC	6	*															*
CC	7																
		*			*												*
CC	8																*
		*															*
CC	9	*															*
		*															*
CT	10							*			*						
		*						*	*								*
CC	11	*						*									*
		*															*
CC	12	*												*			*
CC	13	*						*									
CC	14	*															*
CC	15																*
		*															*
CC	16																*
		*															*
CC	17	*															*
		*															*
CT	18																
		*															*
CC	19																
																	*
CC	20	*															*
		*															*
CC	21																
		*															
CC	22	*															*
		*															
CT	23	*															
																	*
CT	24																
		*															
CC	25	*															*
		*															*
CC	26	*															*
		*															*

C-1562T	Individual	8R	7R	6R	5R	4R	3R	2R	1R	1L	2L	3L	4L	5L	6L	7L	8L
CC	27	*															*
		*															*
CT	28	*				*							*				*
CC	29								*	*							
CC	30							*			*						
CC	31												*	*			
CC	32				*									*			
					*									*			
CC	33										*						
CC	34													*			
CC	35							*									
CT	36													*			
CC	37					*					*						
CC	38							*			*						
CC	39							*			*						
CT	40				*									*			
CC	41							*			*						
CC	42				*												
CT	43						*					*					
CC	44							*			*						
CC	45							*			*						
								*	*	*	*						
CC	46							*			*						
CT	47							*			*						
CT	48							*			*						
CC	49							*			*						
CC	50							*			*						
CC	51							*			*						
CT	52	*															*

Figure 1. Genotypes and profile of permanent dentition in affected individuals. The first column indicates the genotypes of C-1562T polymorphic site. The numbers in the vertical rows represent the tooth group (1 = central incisors; 2 = lateral incisors; 3 = canines; 4 and 5 = 1st and 2nd premolars; 6, 7 and 8 = 1st, 2nd and 3rd molars). L= left quadrant, R= right quadrant. * = missing teeth.

RESULTS

There was no association between the allele frequencies for polymorphism in the MMP-9 gene promoter and hypodontia (p =0.4342). The same was found for the different genotypes (p =0.3236). The C allele was observed at 92.7% and 88.5% frequencies, and the T allele was found at 7.3% and 11.5% frequencies, in the control and test groups, respectively (Table 1). The TT genotype, which is believed to increase MMP-9 transcription, was not observed in either control or test groups. The CT genotype was observed at frequencies of 14.6% and 23% in the control and test groups, respectively, while the CC genotype was observed at frequencies of 85.4% and 77% in control and test groups, respectively (Table 1). The TIMP-2 polymorphism analysis showed that the C allele was not present in the studied population.

DISCUSSION

Gene polymorphisms are a mechanism by which individuals may exhibit variations within the range of what is considered biologically normal. Single nucleotide polymorphisms (SNPs) occur at a high frequency in human genome and can affect gene function. Polymorphisms in the cis-acting sequences of MMPs have been associated with several diseases (14). There is a positive association between the presence of the MMP-9 C-1562T SNP and inflammatory diseases (10). The C-to-T exchange alters the binding of nuclear proteins to this region, leading to increased transcriptional activity in macrophages (10). The expression of MMP-9 is primarily controlled at the transcriptional level, where the promoter of the gene responds to stimuli of various cytokines and growth factors (15). During mouse tooth development, MMP-9 is expressed by mesenchymal cells at bud and cap stages. The expression of this gene is particularly intense around the tip of the growing epithelial organ. Its expression decreases progressively and no transcripts can be detected in dental mesenchyme after the cap stage (8). These results indicate a direct association between the expression of MMP-9 with epithelial morphogenesis. It is worth mentioning that MMP-2 or gelatinase A is widely expressed by mesenchymal cells during tooth development resulting in an overlapping expression of these enzymes at the tip of the growing epithelial bud (8). Therefore, it is possible that the lack of association between the C-1562T polymorphism in the MMP-9 promoter found in the present study occurred due to a functional redundancy between these two MMPs. Gene redundancy is a mechanism devised to assure correct performance of key developmental processes, and it seems to be a frequent phenomena during the early phases of tooth organogenesis (2).

TIMP-2 is widely expressed in dental mesenchyme during early tooth development. Changes in the expression of this molecule could have an influence on dental development. The G-418C polymorphism of the TIMP-2 promoter is located in a Sp1 consensus sequence (16). This polymorphism is located within a 519-bp segment upstream the major transcription initiation site, which was shown to contain active promoter elements (17). It was suggested that the G-418C-nucleotide substitution in the consensus sequence for Sp1 results in down-regulation of the transcription activity of TIMP-2 gene. The high frequency of GG genotype in the TIMP-2 gene promoter makes this site not suitable for studies of DNA polymorphism-disease associations in the studied population (17). However, this polymorphism was associated

Table 1. Distribution of the MMP-9 allele and genotypes in the control and test group.

SNP*	Control Group (without hypodontia)		Test Group (without hypodontia)		p value
	n	%	n	%	
C-1562T allele					
C	89	92.7	92	88.5	0.4342
T	7	7.3	12	11.5	(chi-squared)
Genotype					
CC	41	85.4	40	77	0.3236
TT	0	0	0	0	(Clump)
CT	7	14.6	12	23	

*SNP = single-nucleotide polymorphism.

with the development of chronic obstructive pulmonary disease in a Japanese population (11). Almost thirty percent of the individuals (29.68%) were heterozygous, suggesting that this variant could be common in Oriental subjects. Our results suggest that the frequency of the C allele appears to be very low, or even absent, among Caucasian subjects from the Southeastern region of Brazil.

In addition to elucidate the role of genes in the etiology of alterations of human dentition, the study of mutations/polymorphisms in genes related with tooth development could also help to answer relevant questions of modern biology. One of these questions is how genetic variations interfere with development and are translated into changes in morphology that occur within and among species. Changes in the number of teeth tend to occur in the reverse order that teeth are formed during development, which also characterizes the general pattern of tooth loss observed during the evolution of placental mammals (18). Like hypodontia, most evolutionary changes in tooth number resulted from loss of one or two elements, in most cases the last member of a tooth group. Polymorphic genetic loci have been directly associated with morphological variations in non-vertebrates. As far as it could be ascertained, there is no example of genetic polymorphism that causes morphological variation in vertebrates (19). Studies of DNA polymorphism-hypodontia associations seem to provide an especially suitable model to understand the association of gene polymorphisms and changes in morphology. Teeth are serially homologous structures and the effects of gene variations on their development can be easily quantified (1,2). Individuals with distinct polymorphic alleles may exhibit subtle and specific phenotypic variations in dental patterning. In this sense, studies that investigate possible associations between gene polymorphisms and hypodontia may help to elucidate the genetic mechanisms responsible for the variation in the frequency of tooth agenesis that occur in distinct human populations and ethnic groups (20).

Our data indicate that the C-1562T polymorphism in the MMP-9 promoter is not associated with hypodontia. This lack of association could be a consequence of the sample size. However, the very high p-values found in our statistical analysis are indicative that there is indeed a lack of correlation. It is also possible that changes in the transcriptional activity in the MMP-9 promoter caused by the polymorphism are compensated by other factors acting in the cascade of events that regulate tooth development. Additionally, other polymorphisms present in the coding sequence of MMP-9 and TIMP-2 genes may have an effect on the development of tooth germ.

RESUMO

Hipodontia, a ausência congênita de um ou poucos dentes, é uma das alterações de desenvolvimento mais comuns da dentição humana. Este problema pode causar disfunções na fala e mastigação assim como criar problemas estéticos com implicações ortodônticas e protéticas. A enzima MMP-9 é um membro importante da família das metaloproteinases da matriz, que participa da remodelação da matriz extracelular durante o desenvolvimento do dente. Um polimorfismo genético que consiste em uma troca C-T na posição –1562 cria dois alelos na região promotora deste gene. Os genótipos CT e TT estão relacionados com uma maior atividade deste gene. A proteína inibidora tecidual de metaloproteinase-2 (TIMP-2) regula a atividade das MMPs na matriz extracelular sendo co-expressa com as gelatinases A (MMP-2) e B (MMP-9) durante a morfogênese dental. Um polimorfismo na posição –418 no promotor do gene TIMP-2 foi descrito em um sítio de ligação para o fator de transcrição Sp-1. O objetivo do presente trabalho foi investigar a associação destes polimorfismos genéticos com a hipodontia. A significância das diferenças nas freqüências de polimorfismos observadas entre os grupos teste e controle foi analisada pelo teste de Qui-quadrado ($p<0.05$). Os resultados sugeriram que o polimorfismo na região promotora do gene MMP-9 não está associado com a hipodontia. A alta freqüência do genótipo GG no promotor do gene TIMP-2 demonstrou que este sítio não foi adequado para estudos de associação entre este polimorfismo e doenças no grupo populacional estudado.

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