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# Absence of Association Between Transforming Growth Factor- $\beta$ 1 Promoter Polymorphisms and Hypodontia

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## ABSTRACT

Hypodontia, the congenital absence of one or a few teeth, is one of the most common alterations of the human dentition. The most common permanent missing teeth are the third molars, second premolars, and maxillary lateral incisors. Although hypodontia does not represent a serious public health problem, it may cause masticatory and speech dysfunctions and esthetic problems. Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) is believed to play an important role in tooth development. Its gene is expressed at bud, cap, and bell stages of odontogenesis. Genetic polymorphisms in the TGF- $\beta$ 1 gene promoter were shown to interfere with the transcriptional activity of this gene. To further investigate the role of the TGF- $\beta$ 1 gene in human hypodontia, we analyzed the frequencies of the -509 polymorphism (C-T) alleles and -800 polymorphism (G-A) alleles and genotypes in the TGF- $\beta$ 1 gene promoter in 51 Caucasian subjects with hypodontia and 48 control individuals. Our data suggest that these TGF- $\beta$ 1 promoter polymorphisms are not associated with hypodontia.

**KEY WORDS:** TGF- $\beta$ 1, Hypodontia, Tooth agenesis.

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## INTRODUCTION [Return to TOC](#)


Hypodontia, the congenital absence of one or a few teeth, is one of the most common alterations of the human dentition. Hypodontia can occur as a familial (autosomal dominant, recessive, or X linked) or isolated sporadic trait.<sup>1</sup> The most common permanent teeth missing are the third molars (20%), second premolars (3.4%), and maxillary lateral incisors (2.2%).<sup>2</sup> Although hypodontia does not represent a serious public health problem, it may cause masticatory and speech dysfunctions and esthetic problems. In the past few years, several growth and transcription factors were shown to be expressed in developing teeth.<sup>3,4</sup> The direct participation of several genes in tooth development was evidenced by the lack of teeth in mutant knockout mice models.<sup>5</sup> Autosomal dominant forms of hypodontia were shown to be caused by mutations in the MSX1 and PAX9 genes in human families.<sup>6-11</sup> However, the origin of the isolated sporadic agenesis, the most common form of hypodontia in humans, is still unknown.

Although the exact molecular mechanisms involved in the tissue interactions that regulate tooth development are largely unknown, there is evidence that extracellular matrix (ECM) molecules and growth factors as well as their receptors may play a central role.<sup>12,13</sup> Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) is a signaling molecule that participates in the cascade of signaling events during early tooth development. Its gene is expressed in both epithelia and mesenchyme during bud, cap, and bell stages of odontogenesis.<sup>12</sup> At bud and cap stages, the dental mesenchyme is rapidly proliferating, whereas the dental epithelium intensely expresses TGF- $\beta$ 1 ribonucleic acid (RNA), and the mesenchyme itself also expresses TGF- $\beta$ 1 although at lower levels. Thus, the local expression of TGF- $\beta$ 1 in the dental epithelium may regulate cell proliferation in the underlying dental mesenchyme and contribute to the determination of tooth morphology.<sup>12,14</sup> Another possible function of TGF- $\beta$ 1 during tooth morphogenesis is regulation of ECM deposition. TGF- $\beta$ 1 is known to promote the synthesis of ECM, to modify cell surface matrix receptors, and to prevent degradation of ECM.<sup>15,16</sup>

Gene polymorphisms are mechanisms by which individuals may exhibit variations within the range of what is considered biologically normal. Gene polymorphisms were also shown to be associated with disease susceptibility. Most polymorphisms are single nucleotide exchanges that occur with high frequency in the human genome and may affect the function of genes.<sup>17</sup> Recent evidence indicates that there is a substantial genetic component in the control of total and active TGF- $\beta$  concentration in the circulation. In particular, two polymorphisms identified in the promoter region of the TGF- $\beta$ 1 gene—C-509T and G-800A—were associated significantly with the plasma concentration of TGF- $\beta$ 1.<sup>18,19</sup> Polymorphisms in the promoter region of TGF- $\beta$ 1 gene have been associated with asthma severity,<sup>20</sup> adult periodontitis,<sup>21</sup> renal parenchymal scarring,<sup>22</sup> and Alzheimer disease.<sup>23</sup> The aim of the present study was to test whether the C-509T and G-800A single nucleotide polymorphisms of the TGF- $\beta$ 1 gene are associated with human hypodontia.

## MATERIAL AND METHODS [Return to TOC](#)

### Subject selection and sampling

Fifty-one unrelated individuals with hypodontia and without signs of other disorders ([Table 1](#) ) and 48 healthy control individuals (without hypodontia) were interviewed and the data documented. The congenital absence of teeth was confirmed by X-ray analysis. All subjects were of Caucasian origin and demonstrated no other dental anomalies.

The sampling of epithelial buccal cells was performed as described previously.<sup>24</sup> Briefly, 99 individuals undertook a one-minute mouthwash containing five mL of 3% glucose. After the mouthwash, a sterile wood spatula was used to scrape the oral mucosa. The tip of the spatula was then shaken into the retained mouthwash solution. Buccal epithelial cells were pelleted by centrifugation at 2000 rpm for 10 minutes. The supernatant was discarded and the cell pellet resuspended in 500  $\mu$ L of extraction buffer (10 mM Tris-HCl [pH 7.8], five mM ethylenediaminetetraacetic acid [EDTA], 0.5% sodium dodecyl sulfate [SDS]). The samples were then frozen at -20°C until used for Deoxyribonucleic acid (DNA) extraction. After

having, the samples were incubated overnight with 100 ng/mL proteinase K (Sigma, St. Louis, Mo) at 37°C with agitation. DNA was dissolved in 70 µL of TE buffer (10 mM Tris [pH 7.8], one mM EDTA). The concentration was estimated by measurements of OD 260.

### Polymerase chain reaction and restriction endonuclease digestion

**Polymorphism at position -509.** The oligonucleotides 5' TTTTGCCATGTGCCAGTAG 3' and 5' CACCAGAGAAAGAGACCAG 3' were used as primers. Polymerase chain reactions (PCR) were carried out with 500 ng genomic DNA in a total volume of 50 µL, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, one µM of each primer, 200 µM each deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), and deoxythymidine triphosphate (dTTP), 2.5 mM MgCl<sub>2</sub>, and 2.5 units *Taq* DNA polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden). The reaction was incubated for three minutes at 95°C, followed by 35 cycles of one minute at 95°C, one minute at 58°C, and one minute at 72°C, and a final extension at 72°C for five minutes.

**Polymorphism at position -800.** The oligonucleotides 5' CCCGGCTCCATTCCAGGTG 3' and 5' TGCTCTTGACCACTGTGCCA 3' were used as primers. PCR were carried out with 500 ng genomic DNA in a total volume of 50 µL, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, one µM of each primer, 200 µM each dATP, dCTP, dGTP, and dTTP, 2.5 mM MgCl<sub>2</sub>, and 2.5 units *Taq* DNA polymerase (Amersham Pharmacia Biotech). The reaction was incubated for three minutes at 95°C, followed by 35 cycles of one minute at 95°C, one minute at 62°C, and one minute at 72°C, and a final extension at 72°C for five minutes.




The PCR products were then digested with 1.5 units of the *Eco811* and *MaeIII* restriction enzymes for the analysis of the C-509T and G-800A promoter polymorphisms, respectively. The digest was mixed with 5µL of loading buffer and electrophoresed on a 7.5% vertical nondenaturing polyacrylamide gel. The DNA bands were evidenced by the rapid silver staining method.<sup>25</sup>

### Statistical analysis

The significance of the differences in observed frequencies of polymorphism in both groups was assessed by Chi-squared-test ( $\chi^2$ ). Differences were considered significant when  $P < .05$ . To calculate putative haplotypes and to verify Hardy-Weinberg expectations and linkage disequilibrium, the computer program package ARLEQUIN, version 2.0 was used.<sup>26</sup> The Clump software was used to assess differences in haplotype frequencies 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.<sup>27</sup>

### RESULTS [Return to TOC](#)

In C-509T position, the *Eco811* enzyme cleaves the PCR products into two fragments of 551 and 189 bp, when the polymorphic site contains allele C (but not T), whereas, in G-800A position, the *MaeIII* digests the G allele creating two fragments of 325 and 63 bp.

The statistical analysis did not show significant differences in the alleles and genotypes of both promoter polymorphisms of TGF- $\beta$ 1 gene (C-509T and G-800A) between the two sample groups. In the C-509T polymorphism, the frequency of T allele was 36.4% in the control group and 38.2% in the test group (hypodontia,  $P = .91$ ). The C-T genotype frequency was 43.8% in the control group and 56.9% in the test group ( $P = .41$ ). Regarding the G-800A polymorphism, the allele G was observed with a very similar frequency in both groups (90.6% and 91.2%, respectively;  $P = .91$ ). The genotype G-A was observed in 18.8% of the control group, whereas this same genotype was found in 17.6% of the test group ( $P = 1$ ). The frequencies of different alleles and genotype of the TGF- $\beta$ 1 gene are shown in [Table 2](#) . Comparisons were made with the affected individuals separated into two subgroups including third molar ( $n = 28$ ) and incisor agenesis ( $n = 18$ ), respectively. No significant differences in allele and genotype frequencies were observed when these two groups were confronted or when they were compared with control individuals (not shown). The genotype distribution for the two polymorphisms studied was consistent with the assumption of Hardy-Weinberg equilibrium in both groups. No linkage disequilibrium has been detected. The distribution of the haplotypes arranged as alleles and genotypes were similar between control and test groups ( $P = .92$  and  $P = .55$ , respectively), and the most frequent haplotype in both groups was CG ([Table 3](#) ). The phenotypic and genotypic characterization of the affected individuals is shown in [Table 1](#) .

### DISCUSSION [Return to TOC](#)

TGF- $\beta$ 1 is a pleiotropic cytokine that plays a role in inflammation, tissue repair, as well as in embryonic growth and development.<sup>28</sup> The C-to-T polymorphism at -509 position of the human TGF- $\beta$ 1 promoter creates two different alleles located in a region that is thought to be a negative regulatory area of TGF- $\beta$ 1 gene.<sup>17</sup> This polymorphism creates an YY1 activator consensus sequence.<sup>29</sup> The G-800A polymorphism is located in a region having enhancerlike activity (-1132 to -732), which is thought to overcome the negative regulatory region.<sup>30</sup> Although the role of TGF- $\beta$ 1 in tooth development has not been extensively studied, the expression of its messenger RNA (mRNA) detected by in situ hybridization experiments indicates that this molecule plays a role during the diverse phases of odontogenesis.<sup>12</sup> During the bud and cap stages of mouse molar odontogenesis, TGF- $\beta$ 1 is expressed in dental epithelium and mesenchyme indicating that this factor plays a role in the growth of tooth primordia. Histologic analysis of odontogenesis in TGF- $\beta$ 1 knockout mice showed that the morphogenesis, cytodifferentiation, and histogenesis were unaffected.<sup>31</sup> Although no mRNA for was detected by in situ hybridization, TGF- $\beta$ 1 immunoreactive material was found in the tissues of knockout mice. D'Souza and Litz suggested that maternally-derived TGF- $\beta$ 1 could be responsible for the rescue of developmental events. Unlike the mouse, the development of most human permanent teeth occurs months or years after birth.

A major issue is to understand how genetic variations affect development and are translated into changes in morphology. It is well established that morphological changes that occur among and within species are ultimately determined by genetic interference in the developmental process because small changes during development can produce large changes in adult morphology.<sup>32,33</sup> Experimental studies have linked the spatial and temporal patterns of expression of regulatory genes during development and variations in morphology between species.<sup>32,34-36</sup> Experimental and theoretical approaches have stressed the importance of *cis*-regulatory DNA sequences that control the transcription of genes as the basic modular unit that determines the positional information in developing structures.<sup>37,38</sup> Mutations within individual modules can alter gene transcription in a tissue-specific manner, allowing a mutation to exert its effect on a few morphogenetic fields.<sup>35</sup>

Teeth are becoming a major model in the novel field of evolutionary developmental biology, which links the molecular strategies that control organ development and morphological evolution.<sup>5,39-42</sup> The complexity and dynamics of tooth organogenesis can be exemplified by observing the changes in dental patterning that occurred during the course of vertebrate evolution. Changes in the number of teeth tend to occur in the reverse order of how the teeth are formed during development, which also characterizes the general pattern of tooth loss observed during the evolution of placental mammals.<sup>43</sup> This process could be associated with changes in the regulation of tooth budding morphogenesis. Similar to hypodontia, most evolutionary changes in tooth number resulted from the loss of one or two elements, in most cases the last member of a tooth family. Polymorphic genetic loci have been directly associated with morphological variation in nonvertebrates, and there is to our knowledge no example of genetic polymorphism that causes morphological variation in vertebrates.<sup>44</sup> In this regard, the study of genetic polymorphisms in tooth agenesis is a specially suited model for understanding the association of gene polymorphisms and changes in morphology. Teeth are serially homologous structures, and the effects of gene variations on the development of these structures can be quantified easily.<sup>42,45</sup> Individuals with distinct polymorphic alleles may exhibit subtle and specific phenotypic variations in dental patterning. In this sense, association studies between gene polymorphisms and hypodontia as well as other mild malformations that reflect qualitative defects of embryogenesis<sup>46</sup> may help in understanding the molecular mechanisms that account for the subtle phenotypic variations that characterize distinct human populations and ethnic groups.

Our data indicate that the TGF- $\beta$ 1 promoter polymorphisms are not associated with hypodontia. The lack of association in our results could be a consequence of the sample size. However, the very high  $P$  values found in our analysis are indication that there is indeed a lack of correlation. It is also possible that changes in the transcriptional activity in the TGF- $\beta$ 1 promoter caused by the polymorphisms studied are compensated by other factors in the cascade of events that regulate tooth development.<sup>4</sup> In addition, other polymorphisms present in the coding sequence of the TGF- $\beta$ 1 gene may have a larger effect on the development of tooth germ.<sup>47</sup>

## CONCLUSION [Return to TOC](#)

The results presented in the present study indicate that the -509 (C-T) and -800 (G-A) TGF- $\beta$ 1 promoter polymorphisms are not associated with hypodontia.

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**TABLE 1.** Genotypes and Profile of Permanent Dentition in Affected Individuals<sup>a</sup>

C-509T	G-800A	Upper	8R	7R	6R	5R	4R	3R	2R	1R	1L	2L	3L	4L	5L	6L	7L	8L
		Lower																
CT	GG	1	*															*
CT	AG	2				*												*
CC	GG	3																
CC	GG	4	*															*
CT	AG	5	*															*
CT	GG	6	*															*
TT	GG	7																
CC	AG	8	*			*												*
CT	GG	9	*															*
CC	GG	10	*						*	*		*						*
CT	GG	11	*						*									*
CT	GG	12	*															*
TT	GG	13	*						*						*			
CC	GG	14	*															*
CT	GG	15																*
CT	AG	16	*															*
CT	GG	17	*															*
CT	GG	18	*															*
CT	GG	19																*
CT	GG	20	*															*
CT	GG	21	*															*
CC	GG	22	*															*
CT	GG	23	*															*
CT	GG	24	*															*
CT	GG	25	*															*
CT	GG	26	*															*
CC	GG	27	*															*
CC	AG	28	*				*							*				*
CC	AG	29								*	*							*
CT	GG	30							*		*	*						*
CT	AG	31												*	*			*
CC	GG	32				*	*							*	*			*
TT	AG	33										*						*

TABLE 1. Continued

C-509T	G-800A	Upper Lower	8R	7R	6R	5R	4R	3R	2R	1R	1L	2L	3L	4L	5L	6L	7L	8L
CC	GG	34																
CT	GG	35							*						*			
CT	GG	36																
TT	GG	37					*						*					
CC	GG	38							*				*					
CT	GG	39							*				*					
CT	GG	40				*									*			
CT	AG	41							*				*					
CT	GG	42																
CC	GG	42				*		*					*					
CC	GG	44							*				*					
CC	GG	45							*				*					
CT	GG	46							*	*	*		*					
CT	GG	47							*				*					
CT	GG	48							*				*					
TT	GG	49							*				*					
CC	GG	50							*				*					
CC	GG	51							*				*					

<sup>a</sup> The first two columns represent the genotypes of C-590T and G-800A polymorphic sites. The numbers in the vertical rows represent the position of the tooth (1 central incisors; 2 lateral incisors; 3 canines; 4 and 5 first and second premolars; 6, 7 and 8 first, second and third molars, respectively). L indicates left quadrant; R, right quadrant; \*, missing teeth.

TABLE 2. Distribution of the TGF- $\beta_1$  Allele and Genotype in the Control and Test Group<sup>a</sup>

SNP <sup>a</sup>	Control Group		Test Group		P Value
	n	%	n	%	
<b>C-509T</b>					
Allele					
C	61	63.5	63	61.8	.91 (chi-squared)
T	35	36.5	39	38.2	
Genotype					
C/C	20	41.6	17	33.3	.41 (Fisher exact test)
T/T	7	16.6	5	9.8	
C/T	21	43.8	29	56.9	
<b>G-800A</b>					
Allele					
G	87	90.6	93	91.2	.91 (chi-squared)
A	9	9.4	9	8.8	
Genotype					
G/G	39	81.3	42	82.4	1 (Fisher exact test)
A/A	0	0	0	0	
G/A	9	18.7	9	17.6	

<sup>a</sup> TGF- $\beta_1$  indicates transforming growth factor- $\beta_1$ ; SNP, single nucleotide polymorphism.

**TABLE 3.** Frequency of the Haplotype of the TGF- $\beta_1$  Gene in the Control and Test Group<sup>a</sup>

Haplotype G-800A/ C-509T	Control Group		Test Group		P Value
	n	%	n	%	
Allele					
GC	52	54.2	55	53.9	.92 (clump)
GT	35	36.5	38	37.3	
AC	9	9.4	8	7.8	
AT	0	0	1	1.0	
Genotype					
GC/GT	18	37.5	24	47.1	.55 (clump)
AC/GT	3	6.25	5	9.8	
GC/GC	14	29.2	14	27.5	
AC/GC	6	12.5	3	5.9	
AT/GT	0	0	1	1.9	
GT/GT	7	14.6	4	7.8	

<sup>a</sup> TGF- $\beta_1$  indicates transforming growth factor- $\beta_1$ .

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