

Association of MSX1 and TGFB3 with Nonsyndromic Clefting in Humans

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Summary

Nonsyndromic cleft lip with or without cleft palate (CL/P) and nonsyndromic cleft palate only (CPO) are common congenital anomalies with significant medical, psychological, social, and economic ramifications. Both CL/P and CPO are examples of complex genetic traits. There exists sufficient evidence to hypothesize that disease loci for CL/P and CPO can be identified by a candidate-gene linkage-disequilibrium (LD) strategy. Candidate genes for clefting, including *TGFA*, *BCL3*, *DLX2*, *MSX1*, and *TGFB3*, were screened for LD with either CL/P or CPO in a predominantly Caucasian population, with both case-control- and nuclear-family-based approaches. Previously reported LD for *TGFA* with both CL/P and CPO could not be confirmed, except in CL/P patients with a positive family history. Also, in contrast to previous studies, no LD was found between *BCL3* and either CL/P or CPO. Significant LD was found between CL/P and both *MSX1* and *TGFB3* and between CPO and *MSX1*, suggesting that these genes are involved in the pathogenesis of clefting. In addition, a mutation search in the genes *DLX2*, *MSX1*, and *TGFB3* was performed in 69 CPO patients and in a subset of the CL/P patients. No common mutations were found in the coding regions of these genes; however, several rare variants of *MSX1* and *TGFB3* were found that may alter the latter's normal function. These results form the basis for future research, including (a) mutation searches in the *MSX1* and *TGFB3* genes in Caucasian CL/P patients and (b) extension of the search for *MSX1* mutations in CPO patients to the noncoding regions.

Introduction

Both nonsyndromic cleft lip with or without cleft palate (CL/P [MIM 119530]) and cleft palate only (CPO [MIM 119540]) are common birth defects with characteristics of genetically complex traits. Segregation analysis and epidemiological studies have shown that 25%–35% of CL/P and 10%–20% of CPO patients have a family history of clefting and that simple Mendelian-inheritance models are insufficient to explain the mode of inheritance in families segregating clefting (Fogh-Andersen 1942; Jones 1993; reviewed by Wyszynski et al. 1996). In addition, CL/P and CPO are heterogeneous traits with an estimated 2–20 genes interacting multiplicatively to cause clefts, including a possible major gene that may account for 10%–50% of the incidence of these birth defects (Mitchell and Risch 1992; Fitzpatrick and Farrall 1993; Christensen and Mitchell 1996).

To identify gene(s) involved in CL/P and CPO, investigators have used both association and linkage strategies to evaluate candidate genes, focusing primarily on CL/P. Associations with *TGFA*, *RARA*, *D4S191*, and *BCL3* have been found for CL/P (reviewed by Murray 1995; Wyszynski et al. 1996), and linkage with *BCL3* and 6p has been found for CL/P (Scapoli et al. 1997), whereas an association with *TGFA* has been found for CPO (Shiang et al. 1993; Hwang et al. 1995). Most of the association studies have employed case-control study designs, which are potentially susceptible to population-structure problems. However, family-based study designs that control for the effect of stratification have been developed and can be applied to clefting (Spielman et al. 1993; Thomson 1995). Current technology precludes a genome scan for associations with clefting, thus necessitating the careful selection of candidate genes to study. The advent of gene targeting has led to the identification of several additional candidate cleft genes, including *DLX2* (Qiu et al. 1995, 1997), *MSX1* (Satokata and Maas 1994), and *TGFB3* (Kaartinen et al. 1995; Proetzel et al. 1995).

The purpose of this research was to test the hypothesis

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that TGFA, BCL3, DLX2, MSX1, and TGFB3 are involved in the etiology of CL/P or CPO. This was tested by determining, through the use of both case-control- and nuclear-family-based approaches, whether these genes were in linkage disequilibrium (LD) with either form of clefting. Further testing of the hypotheses was performed by screening CPO patients for mutations in the DLX2, MSX1, and TGFB3 genes.

Subjects and Methods

Populations

An Iowa population that is 95% Caucasian was available to ascertain, by two different mechanisms, cleft lip and cleft palate patients as well as controls. This study has University of Iowa institutional-review-board approval, and informed consent was obtained from all subjects. Initially, a sample of unrelated patients with CL/P ($N = 110$) and CPO ($N = 16$), as well as unmatched controls born during 1956-87, were ascertained by means of a clinic-based mechanism (Ardinger et al. 1989). The second mechanism was a population-based case-control study within the University of Iowa Craniofacial Anomalies Research Center (CARC) (Romitti et al. 1998). Patients with CL/P ($N = 133$) and CPO ($N = 61$), as well as their parents, were ascertained through the Iowa Birth Defects Registry, and control children were selected from birth tapes. For simplification, the first mechanism of ascertainment has been designated "Ardinger," and the second mechanism has been designated "CARC." All patients having a known syndrome or other major or multiple minor defects, as determined by a clinical exam or a record review (by J.C.M. and S.D.-H. [Ardinger et al. 1989]), were excluded during data analysis.

LD Analysis

The cleft patients were separated into two groups, CL/P and CPO, for LD analysis. The case-control comparisons in this study used only CARC controls and compared them with all of the CL/P or CPO patients from both mechanisms of ascertainment. The family-based control analyses were performed only on CARC subjects, since the parental samples were not collected for the first mechanism. The case-control analysis utilizes a larger data set, because not all families were fully ascertained. Two family-based approaches were used, including (1) the affected-family-based control (AFBAC) method, which uses parental and proband genotypes to compare all the transmitted alleles with the untransmitted alleles (Thomson 1995), and (2) the transmission/disequilibrium test (TDT) (Spielman et al. 1993). AFBAC has greater power to detect association than TDT has when no population structure exists, which can be

proved by Hardy-Weinberg equilibrium (HWE) for two generations (Spielman and Ewens 1996). In the presence of population structure, TDT is more powerful, since AFBAC loses power by a larger denominator. However, the size of this underestimation is difficult to predict and may be minimal. Therefore, AFBAC is a more conservative test. In addition, TDT detects linkage in the presence of association and is valid if population structure exists. Both the case-control and the AFBAC data were analyzed by means of $2 \times n$ contingency tables, which were evaluated by either the Pearson χ^2 test; Fisher's exact test, when any of the cells had an expected frequency of ≤ 5 ; or the likelihood-ratio test (LRT) (Terwilliger 1995). Alleles or haplotypes were pooled together when the overall observed frequency for either the sum of the patient and control alleles or the sum of the transmitted and untransmitted alleles was $\leq 5\%$ (Long et al. 1995). The TDT data for multiallelic markers were analyzed by the recently proposed TDT statistic using a $k - 1/k$ correction (where k is the number of alleles), which takes into account the possibility that more than one allele is positively or negatively associated (Spielman and Ewens 1996). Data from families with one parent missing were also used in the TDT analyses (Curtis and Sham 1995; Spielman and Ewens 1996). Bonferroni correction for 95 comparisons would yield a strict $\alpha = .00054$. Haplotypes were determined by GENEHUNTER and were confirmed visually (Kruglyak et al. 1996).

DNA Extraction and Genotyping

DNA was extracted from isolated nuclei of leukocytes, blood spots (Lidral et al. 1997), or buccal mucosa cells (Richards et al. 1993). PCRs were performed in 10- μ l volumes containing 2-4 ng DNA/ μ l; 200 μ M each of dATP, dCTP, dGTP, and dTTP; 1.5 mM MgCl₂; 10 mM Tris/HCl pH 8.3; 50 mM KCl; 0.001% (w/v) gelatin; 0.25-1.0 μ M of each primer; and 0.01-0.02 unit *Taq* polymerase/ μ l. Standard thermocycling was as follows: 94°C for 20-45 s, the primer-specific annealing temperature for 20-45 s, and 72°C for 30 s. Dimethyl sulfoxide (DMSO), 5%-10% (v/v), was added to aid in amplification of GC-rich regions, and the amount of *Taq* polymerase was doubled to compensate for the inhibition of DMSO on *Taq* polymerase.

Markers used include TGFA *Taq*I (Basart et al. 1994), BCL3 CA (St. George-Hyslop et al. 1992), MSX1 CA (Padanilam et al. 1992), and TGFB3 CA (Lidral et al. 1997). An additional marker for TGFA, GGAA4D07 (D4S433), was mapped to the YAC 697h1 containing the TGFA gene. SSCPs for DLX2, MSX1, and TGFB3 were discovered during the mutation screening of the CPO patients. These polymorphisms include DLX2 X2.2; MSX1 X1.1, MSX1 X1.3, MSX1 X2.1, and

MSX1 X2.4; and TGFB3 X5.1 (see Website Lidral et al., AJHG [1998], table 12 and appendixes 2–8; data also are available, on request, from the authors). In addition, a hexanucleotide repeat for TGFB3 5' UTR.1 was identified by the Cooperative Human Linkage Center primer server (Murray et al. 1994). This TGFB3 repeat polymorphism was simultaneously identified by C. Van Broeckhoven (Genome Database accession number 386112), who reported similar allele frequencies.

Subjects were genotyped by means of SSCP, mutation-detection enhancement (MDE), and PAGE techniques. Gels were loaded with either internal controls consisting of samples with known genotypes or a DNA sequencing ladder and were silver stained (Lidral et al. 1997).

Screening for Genetic Variations and Mutations

The genes MSX1 and TGFB3 were screened for mutations in 24 CL/P and 69 CPO patients, including 2 patients with submucous clefts and 1 patient with a bifid uvula, by means of SSCA (Orita et al. 1989) and MDE gels (Soto and Sukumar 1992). A variable number of CPO patients, ranging, for different primer pairs, from 22 to 69, were screened similarly, for mutations in ~50% of the DLX2 coding region. Overlapping primer pairs (see Website Lidral et al., AJHG [1998], appendix 1) were developed to amplify the coding regions, including the splice sites and potential splicing branch sites (Maquat 1996), of DLX2 (J.L.R. Rubenstein, unpublished data), MSX1 (Hewitt et al. 1991), and TGFB3 (Derynck et al. 1988; ten Dijke et al. 1988; Lafyatis et al. 1990).

Sequencing

Sequencing was performed in both directions on DNA samples from two to three individuals, when possible. Templates included either (1) PCR products purified by agarose gels or Qiaquick PCR columns (Qiagen) or (2) asymmetric PCR products generated by use of a primer ratio of 100:1 (20 μ M:0.2 μ M) and were purified by Qiaquick columns. If necessary, the PCR fragments were cloned into pKRX, and 10–20 positive clones were pooled for sequencing (Pruessner et al. 1995). Templates were sequenced by incorporation of either ³³P or dye terminators into cycle-sequencing products, by means of AmpliCycle kits (Perkin-Elmer).

Results

Population Structure

Two anonymous markers chosen at random were evaluated to determine whether there were inherent differences between the patient and control groups. Neither GATA-49C05 nor GATA-51F02 showed any differences between the groups or any evidence of transmission distortion (see Website Lidral et al., AJHG [1998], appen-

dixes 13 and 14). In addition, HWE exists for the control, CL/P, and CPO groups, for all the markers (data not shown). However, it was not possible to prove HWE for two generations in these groups, which would indicate that no population structure exists. TDT is more powerful with population structure, whereas AFBAC is more powerful when no population structure exists (Spielman and Ewens 1996). Therefore, both tests were used—AFBAC because there was no evidence to indicate that structure exists and TDT to improve power if the opposite proved to be the case. Also, AFBAC would underestimate association rather than lead to false-positive findings.

TGFA, BCL3, and DLX2

LD between CL/P or CPO and either the TGFA *TaqI* marker or the GGAA4D07 marker was not detected, by either case-control or family-based approaches (table 1; see also Website Lidral et al., AJHG [1998], appendixes 9 and 10). Also, haplotypes of the *TaqI* and GGAA4D07 polymorphisms did not show evidence of transmission disequilibrium in either the CL/P families or the CPO nuclear families (data not shown). In addition, no evidence of LD between BCL3 or DLX2 and CL/P or CPO was found by family-based analyses (see Website Lidral et al., AJHG [1998], appendixes 9 and 10). No mutations were identified during a screening of the DLX2 coding regions, amplified by the primers X1.3, X1.4, X2.1, X2.2, X3.1, and X3.3, in CPO patients.

MSX1 Mutation Screen and LD

Four polymorphic variants of MSX1 were identified during the mutation screen of the CPO patients. These, along with MSX1 CA variant, were genotyped in the patient and control groups. LD was observed between the CA marker and CPO and between the X1.3 marker and both the CPO and the CL/P groups (table 1).

To address the possibility of population stratification, the data were analyzed by use of nuclear families. Segregation distortion in the control group was not evident for any of the markers (A.C. Lidral, unpublished data). TDT and AFBAC analyses did not show evidence for LD between CL/P and any MSX1 marker (tables 2 and 3). Only the CA marker showed LD with CPO (table 3).

Haplotyping was performed on all the markers, to determine whether LD existed for any haplotype(s). Neither TDT nor AFBAC found LD with any haplotype consisting of markers CA, X1.1, X1.3, X2.1, and X2.4 (table 4). However, a case-control comparison of haplotypes for all five markers showed nearly significant LD with CL/P and CPO (table 5).

A mutation screen of the MSX1 coding region in 69 Iowa CPO patients and 24 CL/P patients revealed nu-

Table 1**Individual Markers: Case-Control LD**

MARKER AND POPULATION	NO. OF CHROMOSOMES OF ALLELE TYPE ^a											P
	1	2	3	4	5	6	7	8	9	10	11	
TGFA GGAA4D07:												
Control	1*	26	8*	37	114	114	65	19 [†]	18 [†]	12*	3*	
CL/P	3 [‡]	25	18 [‡]	43	103	97	53	18 [§]	11 [§]	6 [‡]	0 [‡]	.84
CPO	0	8	2	6	35	32	12	9 [#]	4 [#]	4	1	.66
TGFA <i>TaqI</i> :												
Control	449	53										
CL/P	327	37										.85
CPO	109	15										.62
MSX1 CA:												
Control	64	139	37	310								
CL/P	37	100	19	240								.35
CPO	12	28	1	79								.027 ^b
MSX1 X1.1:												
Control	20	250	60	0	0	0						
CL/P	27	275	67	0	1	0						.80
CPO	7	76	19	0	0	0						.95
MSX1 X1.3:												
Control	5	313										
CL/P	22	372										.005
CPO	8	114										.0057
MSX X2.1:												
Control	3 ^{**}	303	94 ^{**}									
CL/P	1 ^{††}	292	81 ^{††}									.44
CPO	0	88	24									.53
MSX1 X2.4:												
Control	66	182										
CL/P	90	268										.68
CPO	22	78										.37
TGFB3 CA:												
Control	303	161	22									
CL/P	201	127	22									.27
CPO	63	38	5									.85
TGFB3 5' UTR.1:												
Control	453	29										
CL/P	337	17										.45
CPO	98	8										.56
TGFB3 X5.1:												
Control	122	8										
CL/P	156	8										.63
CPO	66	4										1.00 ^b

^a Superscript symbols denote alleles that were pooled.

^b Calculated by the exact test.

merous variants (table 6; see also Website Lidral et al., AJHG [1998], appendices 3-6). These were either polymorphisms or rare variants found in controls, patients, or their parents.

TGFB3 Mutation Screening and LD

The mutation screen of the TGFB3 coding region, excluding exon 4, and of the promotor/5' UTR region, amplified by the primer pair 5' UTR.1, revealed four variants in the Iowa CPO subjects. These occurred in the TGFB3 5' UTR and in exons 2, 5, and 7 (see Website Lidral et al., AJHG [1998], appendixes 7, 8, 11, and 12). The TGFB3 5' UTR.1 and X5.1 variants are poly-

morphisms (table 1), whereas X2.1 and X7.1 are rare variants (table 6; A.C. Lidral, unpublished data). 5' UTR.1 is caused by a hexanucleotide repeat within 50 nucleotides from the transcription initiation sites, and X5.1 is caused by a T→C mutation in intron 4 (table 6).

When a case-control design was used, LD between any TGFB3 marker and either CL/P or CPO was not detected (table 1). The TDT analysis showed LD between CL/P and the TGFB3 X5.1 marker, as well as a trend toward LD between CL/P and the TGFB3 5' UTR.1 marker (table 2). The same analyses did not show LD between CPO and any TGFB3 marker (table 2). There was significant LD between CL/P and the TGFB3 5'

Table 2**TDT LD of Individual Markers**

GENE, MARKER, AND ALLELE	CL/P				CPO			
	Transmitted	Untransmitted	χ^2	<i>P</i>	Transmitted	Untransmitted	χ^2	<i>P</i>
MSX1:								
CA:								
1	13	15			6	5		
2	29	31			8	14		
3	8	8			0	3		
4	36	31	.43	.93	19	9	6.23	.11
X1.1:								
1	9	8			5	2		
2	26	28			13	13		
3	22	21	.10	.95	10	11	.89	.64
X1.3:								
1	8	5	.69	.41	3	2	.20	.65
X2.1:								
1	1	1			0	0		
2	20	26			8	12		
3	24	20	.76	.68	12	8	.80	.36
X2.4:								
1	31	29	.67	.41	11	13	.17	.68
TGFB3:								
CA:								
1	30	35			10	11		
2	25	24			8	7		
3	14	8	1.36	.51	3	3	.08	.80
5' UTR.1:								
1	13	6	2.58	.11	5	4	.11	.75
X5.1:								
1	10	3	3.77	.05	1	2	.33	.58

UTR.1–X5.1 haplotype (table 7). However, in the Iowa population, there was no evidence of LD between any TGFB3 haplotype and CPO (data not shown).

Since there was an association between the TGFB3 5' UTR.1–X5.1 haplotype and CL/P, and since there is evidence for maternal transmission of TGFB1 in mice (Letterio et al. 1994), the data were analyzed to determine whether there was a maternal effect for TGFB3. The TGFB3 5' UTR.1–X5.1 haplotype frequency in mothers of CL/P and CPO patients was compared with that in mothers of controls. No significant difference between the haplotype frequencies was evident (data not shown).

To evaluate whether there was interaction between TGFB3 and MSX1, the frequencies of the MSX1 X1.3 alleles in conjunction with the TGFB3 5' UTR.1–X5.1 haplotypes were compared, between the patients and the controls. There was no evidence for interaction with the CPO phenotype; however, there was a trend ($P = .058$) toward such interaction in the CL/P patients (data not shown). The MSX1 X1.3 allele 1 and the TGFB3 5' UTR.1–X5.1 haplotype 1-1, which were shown to be independently associated with CL/P, were found in combination with each other more frequently in the CL/P chromosomes (7/92) than in the control chromosomes (1/100).

Discussion

TGFA

When two markers were used, there was no significant evidence of LD between TGFA and either CL/P or CPO, in either the case-control- or the nuclear-family-based approach. The differences between these results and those of previous studies of the same population are due to a higher frequency of the rare *TaqI* C2 allele in the current control population than in the control population used in the previous studies: 9.8% in the present study, versus 8.5% (Shiang et al. 1993) and 5.1% (Ardinger et al. 1989) in the previous studies. Also, the C2 allele is less common in affected individuals in the present study: frequency 10% in CL/P patients, versus 13.5% (Ardinger et al. 1989) and 12.6% in CPO patients, versus 19.8% (Shiang et al. 1993). The earlier studies were not population based and relied on convenient control samples, including laboratory workers, placentas, and subjects with other diseases, in contrast to the current study. Two previous reports, using case-control comparisons, have shown LD between TGFA and CPO in Caucasian populations (Shiang et al. 1993; Hwang et al. 1995), and a third almost reached signif-

Table 3

AFBAC LD of Individual Markers

GENE, MARKER AND ALLELE	CL/P ^a				CPO ^a			
	Transmitted	Untransmitted	χ^2	<i>P</i>	Transmitted	Untransmitted	χ^2	<i>P</i>
MSX1:								
CA:								
1	14	15			7*	9*		
2	39	43			11	17		
3	7	8			0*	3*		
4	98	92	.49	.92	36	25	3.07	.04
X1.1:								
1	10	9			4	2		
2	102	105			40	38		
3	26	23	.28	.87	8	12	1.52	.49 ^b
X1.3:								
1	10	7			3	2		
2	158	161	.56	.46	65	66	.21	.65
X2.1:								
1	1 [†]	1 [†]			0	0		
2	109	113			47	51		
3	30 [†]	26 [†]	.35	.55	13	9	.89	.35
X2.4:								
1	40	36			12	15		
2	114	118	.28	.60	48	45	.43	.51
TGFB3:								
CA:								
1	68	73			28	29		
2	41	40			11	10		
3	13	9	.92	.63	3	3	.065	.97
5' UTR:								
1	155	148			53	52		
2	7	14	2.50	.11	5	6	.10	.75
X5.1:								
1	99	92			32	33		
2	3	10	4.02	.08 ^b	2	1	.35	.55

^a Superscript symbols denote alleles that were pooled.

^b Calculated by the exact test.

icance (Shaw et al. 1996), whereas another showed no LD (Stoll et al. 1993). Similarly, LD between TGFA and CL/P has been found in various populations, but not in others, by case-control study designs (reviewed by Wyszynski et al. 1996). There has been considerable variation in study designs, markers used, and percentage of patients with a positive family history, such that direct comparisons are difficult. Also, there is a wide range in the TGFA *TaqI* C2 allele frequency—6.5%–19.8% for CPO, 4%–16.9% for CL/P, and 4%–14% for controls—suggesting that heterogeneity between the populations may exist. Other studies using family-based LD designs have been contradictory (Feng et al. 1994; Maestri et al. 1997; Wyszynski et al. 1997a; Scapoli et al. 1998). A recent meta-analysis of all the published reports showed significant evidence for LD in Caucasian populations (Mitchell 1997). This analysis also revealed significant heterogeneity between the CL/P samples from the different studies, but not in the control samples. The origin of this heterogeneity could not be clearly discerned

from the potential explanatory factors such as population stratification, cleft severity, or percentage of patients with a positive family history of clefting. Population stratification is unlikely, given that the control populations have similar frequencies. Also, the severity of clefts is not significantly different between these studies. One study that did not find LD with CL/P excluded all patients with a positive family history for CL/P (Stoll et al. 1993), suggesting that a family history of clefting may correlate with the TGFA *TaqI* rare variant. The proportion of patients with a positive family history is lower in the present study's data set than in previous studies of this population (Ardinger et al. 1989), which may explain the current lack of LD. The importance of a positive family history is demonstrated in the present study by the significant transmission of the rare allele ($P = .014$) to CL/P patients with a positive family history (P.A. Romitti and J.C. Murray, unpublished data). However, two previous studies (Hecht et al. 1991; Vintiner et al. 1992) did not reveal linkage between TGFA

Table 4

MSX1 CA-X1.1-X1.3-X2.1-X2.4 Haplotype: TDT and AFBAC Analysis

POPULATION AND HAPLOTYPE	TDT				AFBAC			
	Transmitted	Untransmitted	χ^2	P	Transmitted	Untransmitted	χ^2	P
CLP:								
1-2-2-2-2	6	7			5*	8*		
1-2-2-3-2	1	0			1 [†]	0 [†]		
1-3-2-2-2	0	2			0 [†]	2 [†]		
2-2-2-2-2	14	18			18	22		
2-2-2-3-2	0	1			0 [†]	1 [†]		
2-4-2-2-2	0	1			0 [†]	1 [†]		
3-2-1-2-2	1	0			1 [†]	0 [†]		
3-2-2-2-2	5	4			4*	3*		
3-2-2-3-2	0	2			0 [†]	2 [†]		
4-1-2-2-2	0	1			0 [‡]	1 [‡]		
4-1-2-3-1	5	6			5 [§]	5 [§]		
4-2-1-2-2	3	2			4 [‡]	3 [‡]		
4-2-2-1-2	1	0			1 [‡]	0 [‡]		
4-2-2-2-2	18	13			24	23		
4-2-2-3-1	1	1			1 [‡]	1 [‡]		
4-2-2-3-2	1	1			0 [‡]	1 [‡]		
4-3-2-2-1	7	4			7 [§]	0 [§]		
4-3-2-2-2	1	0			1 [‡]	0 [‡]		
4-3-2-3-1	6	7	12.97	.74	8	7	5.65	.47 ^b
CPO:								
1-2-2-2-2	4	3			2*	3*		
1-2-2-3-2	1	0			1 [†]	0 [†]		
1-3-2-2-2	1	1			1*	1*		
2-2-2-2-2	6	14			6	14		
2-2-2-3-2	1	0			1 [†]	0 [†]		
3-2-2-2-2	0	2			0 [†]	1 [†]		
3-3-2-3-1	0	1			0 [†]	1 [†]		
4-1-2-2-1	0	1			0 [‡]	1 [‡]		
4-1-2-3-1	0	1			0 [‡]	1 [‡]		
4-2-1-2-2	2	1			2 [‡]	1 [‡]		
4-2-2-2-2	12	6			13	5		
4-2-2-3-2	1	0			1 [†]	0 [†]		
4-3-2-2-1	3	2			2 [‡]	2 [‡]		
4-3-2-3-1	4	3	13.00	.45	3	2	7.10	.28 ^b

^a Superscript symbols denote alleles that were pooled on the basis of at-risk allele 4 of the CA marker.

^b Calculated by the exact test.

and CL/P in small collections of multiplex families, which underscores the heterogeneity of clefting.

A role for TGFA in clefting is not supported by data from transgenic mice, regardless of whether the latter are ectopically expressing or null mutants (Jhappan et al. 1990; Sandgren et al. 1990; Luetke et al. 1993; Mann et al. 1993). Transgenic knockout or dominant-negative mice mutant for EGFR, the receptor for TGFA, also have normal craniofacial development (Murillas et al. 1995; Sibilia and Wagner 1995; Threadgill et al. 1995).

Recently, an additive association of the TGFA *TaqI* C2 allele and smoking has been shown, with CPO, in both a Maryland population (Hwang et al. 1995) and in an Iowa population (P.A. Romitti and J.C. Murray, unpublished data), and with both CL/P and CPO, in California and Mid-Atlantic populations (Shaw et al.

1996; Maestri et al. 1997). In addition, certain TGFA genotypes and alcohol exposure have been shown to increase risk for CL/P (P.A. Romitti and J.C. Murray, unpublished data). These data suggest that TGFA may modulate susceptibility to environmental exposures.

BCL3 and DLX2

No evidence for LD between BCL3 and either CL/P or CPO was detected, in contrast to the results reported in previous studies (Stein et al. 1995; Amos et al. 1996; Maestri et al. 1997; Wyszynski et al. 1997b). The data from the present study do not show evidence for LD between DLX2 and either CL/P or CPO. Furthermore, no mutations were found in a partial screen of the DLX2 coding region in CPO patients. The phenotype of the mouse knockout indicates that a similar mutation in

Table 5**MSX1 CA-X1.1-X1.3-X2.1-X2.4 Haplotype: Case-Control LD**

HAPLOTYPE	NO. OF CHROMOSOMES ^a		
	Control	CL/P ^b	CPO ^c
1-2-2-2-2	18*	10	6
1-2-2-3-2	0*,†	1*	1†
1-3-2-2-2	2*,†	0*	1†
2-2-2-2-1	2*,†	0*	0†
2-2-2-2-2	57	24	14
2-2-2-3-2	0*,†	1*	1†
2-3-2-2-2	0†	0	1†
3-2-1-2-2	0*	2*	0
3-2-2-2-2	16	6	0
4-1-2-2-1	0†	1*	0
4-1-2-3-1	10 [§]	6	0 [§]
4-2-1-2-2	3 ^{‡,§}	4 [‡]	2 [§]
4-2-2-1-2	4 ^{‡,§}	1 [‡]	0 [§]
4-2-2-2-2	52	41	19
4-2-2-3-1	4 ^{‡,§}	1 [‡]	2 [§]
4-2-2-3-2	0 ^{‡,§}	1 [‡]	2 [§]
4-3-2-2-1	0 ^{§,}	9	3 [§]
4-3-2-2-2	0 ^{‡,§}	1 [‡]	2 [§]
4-3-2-3-1	27	10	4
4-3-2-3-2	1 ^{‡,§}	0 [‡]	0 [§]
4-5-2-2-2	0 [‡]	1 [‡]	0
Total	196	120	58

^a Superscript symbols denote alleles that were pooled on the basis of at-risk allele 4 of the CA marker.

^b $\chi^2 = 12.50$; $P = .052$ (by the exact test).

^c $\chi^2 = 13.04$; $P = .069$ (by the exact test).

humans may result in a cleft palate along with other craniofacial anomalies (Qiu et al. 1995).

MSX1 Mutation Screening and LD

LD was found between both CL/P and CPO and the MSX1 X1.3 marker, a neutral polymorphism, when patient and control populations were compared. In addition, LD between CPO and the MSX1 CA marker exists. The LD between MSX1 X1.3 and CL/P or CPO, observed by the case-control-based analysis, was not confirmed by the nuclear-family-based analyses. There was no evidence of LD between any of the other MSX1 markers and CL/P. However, significant LD between the MSX1 CA and CPO was confirmed by the AFBAC analysis.

Haplotype comparison of the CL/P or CPO probands with the controls showed evidence of nearly significant LD with the 4-2-2-2-2 and 4-3-2-2-1 haplotypes, suggesting that they may carry a mutation involved in the etiology of CL/P and CPO. The 4-2-2-2-2 haplotype represents the association of CA allele 4 and the common alleles for the other markers, which correspond with the published sequences. The 4-3-2-2-1 haplotype, which is not found on any of the control chromosomes, contains an amino acid substitution, Ala34Gly. This substitution may affect a series of alanine residues in the N-terminal

region which may be important for normal function (Mortlock et al. 1996; Zhang et al. 1996). Analysis of the haplotype data for the CL/P and CPO patients did not show significant LD with haplotypes of all five markers, either by case-control- or family-based analyses (tables 4 and 5). However, the same haplotypes—4-2-2-2-2 and 4-3-2-2-1—were transmitted more frequently to the affected probands.

The entire MSX1 coding region was screened in 69 CPO patients and 24 CL/P patients from Iowa. In addition, this region was screened, by means of the MSX1 X1.1, MSX1 X1.3, MSX1 X2.1, and MSX1 X2.4 primers, in a much larger number of CL/P patients during the genotyping process. No obvious mutations were identified that would alter the known function of MSX1, including the function of either the homeodomain or a potential phosphorylation site upstream of the homeodomain, by a cAMP-dependent phosphorylation protein kinase. Therefore, it is unlikely that mutations in these elements are common in CPO patients. However, a number of variants of unknown effect were identified, which in the future may be shown to have an etiologic role by

Table 6**Sequence Summary of MSX1 and TGFB3 Variants**

Gene, Marker, and Allele	Mutation ^a
MSX1:	
X1.1:	
1	403 G→A, 5' UTR
2	Published sequence
3	539 C→G, Ala34Gly
4	506 C→T, Ala23Val (two mothers of cases)
5	404 G→C, Gly12Arg in 5' UTR open reading frame (case from mother)
6	528 C→A, Ala31Glu (control father)
X1.3:	
1	768 C→T, Gly110Gly
X2.1:	
1	2081 G→A, Leu181Leu, and T insertion between positions 1968 and 1975
2	T insertion between positions 1968 and 1975
3	Published sequence
X2.4:	
1	2438 C→T, 3' UTR
TGFB3 5:	
5' UTR.1:	
2	Δ at -1083 to -1086 (AGAGGG repeat)
X2.1:	
1	383 A→G, Lys128Arg
X5.1:	
1	T→C, at position -24 relative to intron 4/exon 5 junction
X7.1:	
1	1116 C→G, Ala372Ala

^a For MSX1 markers, the nucleotide numbers correspond to those of Hewitt et al. (1991); for TGFB3 markers, the nucleotide numbers are relative to the translation-initiation codon (Derynck et al. 1988; ten Dijke et al. 1988; Lafyatis et al. 1990).

Table 7**TGFB3 Haplotypes for Markers 5' UTR.1 and X5.1: TDT and AFBAC Analysis in CL/P Patients**

Haplotype	TDT				AFBAC			
	Transmitted	Untransmitted	χ^2	<i>P</i>	Transmitted	Untransmitted	χ^2	<i>P</i>
1-1	17	6			90	79		
1-2	2	9			2*	9*		
2-1	4	7			4*	8*		
2-2	0	0	7.02	.03	0	0	5.81	.008 ^a

^a LRT statistic.

a) altering the protein function or b) affecting splicing through alterations in exonic splicing elements, which have been described in other genes (Cooper and Mattox 1997). If these variants do not affect MSX1 function, then it may be necessary to extend the mutation search to enhancer regions shown to regulate MSX1 in mice (MacKenzie et al. 1997).

It has been proposed that cleft palate in *Msx1* knockout mice is due to insufficient palatal mesenchyme, which, in turn, is due to the lack of dental development (Ferguson 1994). This is supported by a higher frequency of anodontia in cleft patients than in controls (Ranta 1986). A family with partial anodontia and a mutation in the MSX1 homeobox has been described elsewhere (Vastardis et al. 1996). None of the family members had a cleft lip or cleft palate. However, the maxilla of the proband was described as being slightly smaller, although within the normal range. It is possible that the MSX1 mutation may also be affecting the mid-face region, but not severely enough to result in a cleft palate. Alternatively, the small maxilla may be a characteristic of this family that is independent of the MSX1 mutation, since this is a relatively common trait in humans. This suggests that, for null MSX1 mutations to be associated with palatal clefts, complete anodontia may need to occur. We plan to correlate anodontia and the MSX1 genotypes, in both cleft and control subjects.

TGFB3 Mutation Screening and LD

Despite the wealth of data from expression assays (Fitzpatrick et al. 1990; Pelton et al. 1990), transgenic knockout mice, (Karttinen et al. 1995; Proetzel et al. 1995), and inactivation of TGFB3 by antisense oligonucleotides and antibodies (Brunet et al. 1995), which indicate that TGFB3 has a crucial role in secondary palate development, there is no genetic evidence for this in the Iowa population. This may suggest that the lack of palate fusion, especially that which is due to TGFB3 mutations, is not a common cause of CPO in humans, indicating that the developmental etiology of secondary palate clefts may be different in humans than it is in mice. However, it is possible that numerous mutations

have arisen on different backgrounds, in which case LD would not be detected but linkage could exist.

A mutation screen of the Iowa CPO population was performed under the hypothesis that, despite the lack of evidence that TGFB3 plays a role at the population level, the considerable biological evidence for the role of TGFB3 in secondary palate development supports the hypothesis that either a small number of individuals may have a causal mutation in the coding region of the gene, or heterogeneity may have prevented LD detection. Two rare variants were identified in two CPO individuals. The first of these rare variants, TGFB3 X7.1, is a neutral mutation and was transmitted from the mother, who is phenotypically normal, whereas the father, who does not have the variant, has CPO, indicating that this variant is not etiologic for CPO. The second rare variant, TGFB3 X2.1, results in a conservative amino acid change, Lys128Arg, in the protein-precursor region. This X2.1 variant may be a rare, functionally neutral variant; however, it has not been identified in almost 700 control chromosomes ($P = .023$), and this amino acid is conserved in mammals (Denhez et al. 1990). The subject who was heterozygous for this variant has a submucous cleft palate. TGFB3 null mice have been described as having a similar phenotype in situations in which fusion has occurred (Proetzel et al. 1995). In this family, the variant was maternally transmitted. Interestingly, the mother and some of her relatives have scarring of the lip, which apparently is due to spontaneous in utero repair of a cleft lip (MIM 600625), which is a rare occurrence (Castilla and Martinez-Frias 1995).

The identification of only two coding variants in CPO patients indicates that mutations of the protein are not common in this population. Other potential sites for mutations include the small fourth exon, the regulatory regions in the promoter, and the 5' and 3' UTRs.

The LD between TGFB3 and CL/P was unexpected, given the lack of a similar phenotype in the knockout mouse and the lack of LD with CPO in humans. This is supported by the recent report of LD with D14S61 in nuclear families that have either CPO or CL/P (Maestri et al. 1997). The lack of LD between CL/P and the TGFB3 CA marker could occur if several mutations

arose on different CA alleles, whereas, for the other two TGFB3 markers, mutations always arose on the more common alleles.

There was no evidence for a maternal effect, with either CL/P or CPO, when the TGFB3 5' UTR.1-X5.1 haplotype was used. A precedent for this exists in TGFB1 in mice (Letterio et al. 1994). It is unknown whether TGFB3 can act similarly, but the presence of a cleft-palate phenotype in null embryos from hemizygous dams (Kaartinen et al. 1995; Proetzel et al. 1995) indicates that TGFB3 may be different from TGFB1 in this respect.

There was a suggestion of interaction between the MSX1 and TGFB3 loci, in the etiology of CL/P. A combination of alleles was observed in 7.6% of the CL/P patients and in 1.0% of the controls. Therefore, this combination may explain a significant proportion of CL/P patients, in light of the hypothesized relative risks for the etiologic genes.

The expression of TGFB3 has not been described during primary palate formation, which also involves fusion. It may be that TGFB3 is expressed in the epithelium of the developing nasal and maxillary processes in the area of future fusion. The absence of a cleft-lip phenotype in mice lacking TGFB3 may be due to strain differences in susceptibility to cleft lip versus cleft palate. The data for human CL/P and TGFB3 warrant screening of CL/P patients for causal mutations in the gene. This is also supported by an increased risk for CL/P and CPO when smoking and/or alcohol exposure occur in conjunction with certain TGFB3 genotypes (P.A. Romitti and J.C. Murray, unpublished data), which recently has been reported (Maestri et al. 1997).

Strict interpretation of statistical significance would indicate that the LD data presented here could be due to chance. Yet, the data must be interpreted in light of other biological data supporting a higher prior probability of an etiologic role. Final interpretation will be facilitated by replication in additional populations.

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Electronic-Database Information

Genome Database, <http://www.ncbi.nlm.nih.gov> (for TGFB3 repeat polymorphism [accession number 386112])
Lidral et al., *AJHG* (1998), http://genetics.uiowa.edu/~alidral/AJHG_article-1998
Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/omim> (for CL/P [MIM 119530], CPO [MIM 119540], and cleft lip [MIM 600625])

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