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Cleft Lip With or Without Cleft Palate: Associations with Transforming Growth Factor Alpha and Retinoic Acid Receptor Loci

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Summary

The first association study of cleft lip with or without cleft palate (CL/P), with candidate genes, found an association with the transforming growth-factor alpha (TGFA) locus. This finding has since been replicated, in whole or in part, in three independent studies. Here we extend our original analysis of the TGFA *Taq*I RFLP to two other TGFA RFLPs and seven other RFLPs at five candidate genes in 117 nonsyndromic cases of CL/P and 113 controls. The other candidate genes were the retinoic acid receptor (RARA), the *bcl*-2 oncogene, and the homeobox genes 2F, 2G, and EN2. Significant associations with the TGFA *Taq*I and *Bam*HI RFLPs were confirmed, although associations of clefting with previously reported haplotypes did not reach significance. Of particular interest, in view of the known teratogenic role of retinoic acid, was a significant association with the RARA *Pst*I RFLP ($P = .016$; not corrected for multiple testing). The effect on risk of the A2 allele appears to be additive, and although the A2A2 homozygote only has an odds ratio of about 2 and recurrence risk to first-degree relatives (λ_1) of 1.06, because it is so common it may account for as much as a third of the attributable risk of clefting. There is no evidence of interaction between the TGFA and RARA polymorphisms on risk, and jointly they appear to account for almost half the attributable risk of clefting.

Introduction

Cleft lip with or without cleft palate (CL/P) is one of the most common congenital anomalies, with an incidence of 1/700–1/1,000 live births among Caucasians. Approximately 20% of patients have a positive family history of CL/P, and thus genetic factors are thought to be important in its etiology. While some of the nonsyndromic but familial cases have a clear Mendelian pattern of inheritance (Rollnick and Kaye 1986), the majority appear to be inherited in a more complex manner (Melnick et al. 1980). Several claims of major gene effects have been made (Marazita et al. 1984, 1986; Chung et al. 1986; Hecht et al. 1991*b*), but recent reanalyses by Farrall and Holder (1992) and by Mitchell and Risch (1992) suggest that none of these data are incompatible with multifactorial, or at least oligogenic, inheritance. Analysis of patterns of recurrence risk in relatives in these latter two studies suggests that a fairly large number of genes are acting multiplicatively (epistasis) and that the gene of largest effect could only account for an increased risk of about sixfold in first-degree relatives, compared with an observed value of about 30-fold.

While the genetic epidemiology of clefting has, for decades, been the subject of considerable research, until recently there was no information about specific genes that might be involved. In 1989, Ardinger et al. (1989) published the first association study of CL/P with five candidate genes chosen because there was evidence from rodents that they were involved in palate formation. Analysis of 80 unrelated patients from Iowa showed that there were significant associa-

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tions ($P = .005$) of CL/P with *Taq*I and *Bam*HI RFLPs at the transforming growth factor alpha (TGFA) locus. The association with the TGFA *Taq*I RFLP has since been replicated in two different populations; the rare C2 allele was found in 17.2% of chromosomes from Australian CL/P patients but in only 5.5% of control chromosomes ($P = .0003$; Chenevix-Trench et al. 1991), and, in an English sample, it was found in 21% of CL/P chromosomes compared with 4% of control chromosomes ($P < .001$; Holder et al. 1992). In contrast, the association with the *Taq*I polymorphism was not confirmed in an Alsacian population of nonfamilial clefting, although an association with a different *Bam*HI RFLP of TGFA with bilateral CL/P was found (Stoll et al. 1992).

Here we extend our original analysis of the *Taq*I TGFA RFLP in 96 CL/P patients and 100 controls (Chenevix-Trench et al. 1991), to include two other TGFA RFLPs (*Bam*HI and *Hin*fI), as well as seven other RFLPs at five new candidate genes—retinoic acid receptor (RARA), *bcl*-2 oncogene, and the homeobox genes 2F, 2G, and EN2.

All-*trans* retinoic acid (RA), a naturally occurring form of vitamin A (retinol), is a recognized teratogen for cleft palate (Geelen 1979). Retinol is essential for normal development and RA, to which retinol can be metabolized, may be a morphogen during embryogenesis (Satre and Kochhar 1988). We hypothesized that variation in genetic susceptibility to RA may result from variation at the RARA locus, with concomitant effects on the target genes that this nuclear protein activates, including homeotic genes (Simeone et al. 1990). Sulik et al. (1988) have suggested that RA may act, at least in part, by altering the timing and amount of apoptosis during embryogenesis. Precise control of apoptosis is essential for correct formation of the lip and palate, and it is possible that variation in genes involved in apoptosis may be directly responsible for some of the predisposition to CL/P. We therefore examined *bcl*-2, a gene that codes for a mitochondrial protein that blocks apoptosis (Hockenbery et al. 1990). The homeotic genes are also candidates for involvement in clefting, being transcription factors expressed in an organ- and stage-specific manner. More than 30 human homeobox genes have been cloned and found to be clustered together in four HOX loci on chromosomes 7p (HOX 1), 17q (HOX 2), 12q (HOX 3), and 2q (HOX 4) (Acampora et al. 1989). There are other homeobox-containing genes, such as the *engrailed*-like gene (EN2) (Logan and Joyner 1989*a*, 1989*b*) and the putative aniridia (AN) gene (Ton et al. 1991), which map outside these clusters. Remarkably few RFLPs have been described in homeobox genes; we chose to study those that were most easily detected at a reasonable cost.

Subjects and Methods

Patients and Controls

Blood samples were collected from 117 unrelated nonsyndromic patients and 113 unrelated controls. All these individuals were genotyped for the TGFA *Taq*I RFLP, and 41–115 patients and 43–112 controls were genotyped for the other RFLPs. The exact number of individuals who were genotyped for each RFLP depended largely on DNA availability. Of the patients, 71 (61%) were male, and 65 (59%) of the 110 for whom the information was available reported a family history (in first-, second-, or third-degree relatives) of CL/P. This high percentage of patients with positive family history probably reflects our ascertainment method, which was mainly through newspaper articles in which we encouraged participation from familial cases. There were 88 (75%) patients with a cleft lip and cleft palate, of whom 21 (24%) were bilateral cases, 17 (19%) were right sided, 49 (56%) were left sided, and 1 was unknown. The remaining 29 patients had isolated cleft lips, of which 2 (7%) were bilateral, 5 (17%) were right sided, and 22 (76%) were left sided. This excess of left-sided clefts has been observed by others (Holder et al. 1992). In order to determine that the patients were nonsyndromic, a clinical examination—with particular attention to lip pits, ear anomalies, and limb anomalies—was carried out, and the patients were asked specific questions about neonatal respiratory problems.

The controls, all from Australia, were of unknown clefting status and comprised the following groups: 33 geriatric patients, 34 laboratory workers, 27 spouses of patients with inherited disorders, and 19 mothers of twins. All patients and controls were of Caucasian extraction, the majority being of Anglo-Celtic descent, and, in particular, all four grandparents of all patients were reported to be Caucasian. Ethnicity of grandparents was not available for the controls.

Southern Blot Analyses

DNA was isolated from peripheral blood lymphocytes or lymphoblastoid cell lines by the salting out

method (adapted from Miller et al. [1988]). Ten micrograms of DNA was digested with restriction enzymes in the presence of 4 mM spermidine, prior to separation in 0.6%–1.1% agarose for 18–65 h. DNA was transferred to Hybond N + in 0.4 M NaOH (Reed and Mann 1985). Plasmids were radioactively labeled by random priming and hybridized to Southern filters at 65°C in 2 × SSPE, 1% SDS, 0.5% Blotto, 0.5 mg salmon sperm DNA/ml with or without 10% dextran sulfate. The filters were washed to a stringency of 0.5 × SSC at 65°C. The plasmids used are described in table 1. The *Hin*fI TGFA RFLP was analyzed instead of the *Rsa*I RFLP, with which it was in complete disequilibrium in the 30 individuals for whom both genotypes were obtained, because we found the *Hin*fI digestions to be more reliable. In the course of this work, two new alleles were discovered at the TGFA and RARA loci (figs. 1 and 2). For the purposes of analysis, these were combined with the other rare alleles at these loci.

Statistical Analysis

Contingency table analysis, with significance calculated by Fisher exact test or estimated by χ^2, was used to assess allele, genotype, and haplotype frequency differences between cases and controls. To assess potential interaction between risk factors and to estimate odds ratios (ORs) and attributable risks, the exact methods for combining 2×2 tables of Thomas and Gart (1992) were used.

Heritability due to single-gene effects was calculated from the disease prevalence and relative risk as follows. Let a denote the probability that both members of a pair of relatives are affected, and let $2b$ denote the probability that the pair is discordant. Then the prevalence of the trait (K) is $(a + b)$, and the relative risk $\lambda = a/K^2$, so $a = \lambda K^2$. The bivariate normal distribution (Kirk 1973) was then evaluated to solve for the corresponding tetrachoric correlation ρ (as $a = \Phi[\Phi^{-1}(K), \Phi^{-1}(K), \rho]$). The narrow heritability is then ρ/coefficient of relationship. One estimate of

Table 1

RFLPs Examined

Locus	Probe	Location	RFLP	Allele (size)	Reference
TGFA	phTGF1-10-925	2p13	*Taq*I	C1 (3.0 kb) C2 (2.7 kb)	Hayward et al. 1987
TGFA	phTGF1-10-3350	2p13	*Bam*HI	A1 (11.0 kb) A2 (7.2 kb) A3 (15.0 kb)	Murray et al. 1986
TGFA	phTFG1-10-3350	2p13	*Hin*fI[a]	D1 (2.5 kb) D2 (2.0 kb)	J. Murray, personal communication
EN2	mp4	7q36	*Pvu*II	A1 (1.2 kb) A2 (1.0 kb)	Logan and Joyner 1989*a*
EN2	mp5	7q36	*Sac*I	A1 (11 kb) A2 (6.8 kb)	Logan and Joyner 1989*b*
RARA	ph-RARα	17q21.1	*Pst*I	A1 (3.0 kb) A2 (2.6 + 0.4 kb)	Arveiler et al. 1988
HOX2F ...	ph2-1-3Bg	17q21-22	*Msp*I	A1 (2.0 kb) A2 (1.8 kb)	Ogura et al. 1991*a*
HOX2G ...	ph2-1-9	17q21-22	*Taq*I	A1 (3.8 kb) A2 (3.2 kb)	Ogura et al. 1991*b*
HOX2G ...	ph2-1-9	17q21-22	*Msp*I	B1 (3.9 kb) B2 (2.0 kb)	Ogura et al. 1991*b*
BCL2	pB16	18q21.3	*Eco*RI	A1 (10 kb) A2 (20 kb)	Tsujimoto et al. 1987

[a] In complete linkage disequilibrium with the *Rsa*I RFLP reported by Murray et al. (1986).

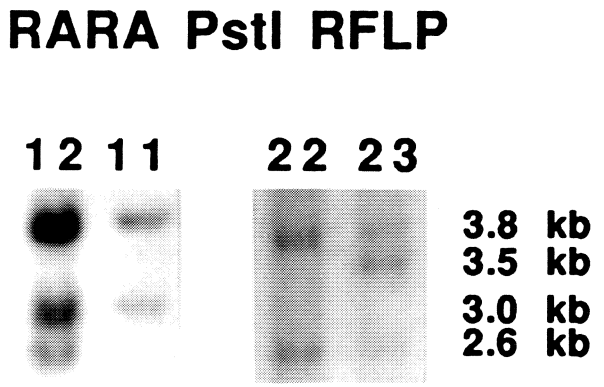

Figure 1 RARA *Pst*I RFLP. Four genotypes from CL/P cases demonstrating the A1 (3.0 kb), A2 (2.6 kb), and A3 (3.5 kb) alleles and a constant band at 3.8 kb.

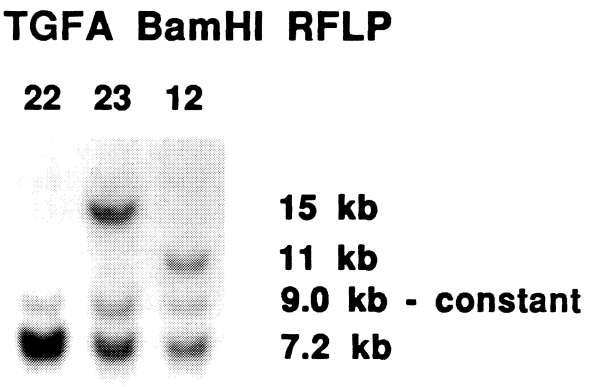

Figure 2 TGFA *Bam*HI RFLP. Three genotypes from CL/P cases demonstrating the A1 (11 kb), A2 (7.2 kb), and A3 (15 kb) alleles and a constant band at 9.0 kb.

the broad heritability is simply ρ_{MZ}, in the absence of evidence for shared environmental effects (Smith 1970). A BASIC program for evaluation of these and ancillary statistics for the biallelic case is available on request from D. L. Duffy.

Results

The results of genotyping CL/P cases and controls for 10 RFLPs are given in table 2. At each locus, genotype frequencies among controls did not differ significantly from Hardy-Weinberg equilibrium. There were no significant allele-frequency differences between CL/P cases and controls, at the EN2, HOX2F, HOX2G, and BCL2 loci ($P > .05$). In the analysis of the TGFA *Taq*I RFLP, data from 63 Australian controls reported by Hayward et al. (1988) were included, since there was no significant difference between the allele frequencies in those controls and those in the 113 new controls genotyped by us ($P = .54$). Using a two-tailed test, we found a significant association of CL/P with the *Taq*I RFLP at the TGFA locus ($P = .005$), but not with the *Hin*fI or *Bam*HI RFLPs ($P = .23$ and $P = .053$, respectively), although the latter is significant when a one-tailed test is used ($P = .029$), which is justifiable in view of the previous finding by Ardinger et al. (1989). The C2 *Taq*I allele was found in 15% of CL/P chromosomes and in 9% of control chromosomes, while the A2 *Bam*HI allele was found in 92% of cases and 86% of controls.

There was also a significant difference between cases and controls in the frequency of the A2 *Pst*I allele at the RARA locus ($P = .016$; two-tailed test), occurring in 79% of CL/P chromosomes and 67% of control chromosomes, although the association was not significant when the rejection criterion was adjusted for the seven new test comparisons made (alpha = $0.05 \div 7 = 0.007$). Genotype distributions were heterogeneous between cases and controls ($\chi^2_2 = 6.5$, $P = .038$), and this appeared to be mainly due to an excess of affected A2A2 homozygotes. Among those with a positive family history, 39/58 (67%) were A2A2 RARA homozygotes, compared with 26/45 (57%) with a negative family history ($P = .41$). Of those with cleft lip alone, 14/27 (52%) were A2A2 homozygotes, compared with 56/83 (67%) of those with cleft lip and palate ($P = .17$).

For both the TGFA *Taq*I and *Bam*HI RFLPs, and for the RARA *Pst*I RFLP, there were, among cases, no significant associations between the associated allele and sex, family history, type of cleft (CL/P vs. cleft lip alone), or location of cleft (bilateral vs. unilateral or left vs. right sided). Of the 65 cases with a positive family history, only 19/65 (29%) carried the C2 TGFA *Taq*I allele, while 63/64 (98%) carried the A2 TGFA *Bam*HI allele. Among the controls, 11/45 (24%) carried the C2 *Taq*I allele, and 44/44 (100%) carried the C2 *Bam*HI allele. Of 58 cases genotyped for the RARA RFLP who had a positive family history, 93% carried the A2 allele, as did 93% of controls. There was a nonsignificant tendency for the A2 *Pst*I RARA allele to occur more frequently in cases with cleft lip and cleft palate than in cases with isolated cleft lip ($P = .055$).

Haplotype frequencies were only obtained for the TGFA locus, since neither of the two RFLPs analyzed at the HOX2G locus showed a significant association with CL/P. Haplotypes could be assigned for 90/109

Table 2

Genotype and Allele Frequencies in CL/P Patients and Controls

Locus	RFLP	Group	N	No. of Individuals with Genotype					Frequency of Allele			*P* Value[a]
				11	12	22	13	23	1	2	3	
TGFA	*Bam*HI	CL/P	115	1	14	97	0	3	.070	.917	.013	.053[b]
		Controls	112	0	26	82	2	2	.125	.857	.018	
RARA	*Pst*I	CL/P	110	7	32	70	0	1	.218	.777	.005	.016[b,c]
		Controls	75	8	33	34	0	0	.327	.673	.000	
				11	12	22			1	2		
TGFA	*Taq*I	CL/P	117	84	30	3			.846	.154		
		Controls	113	94	17	2			.907	.093		.049[c]
			176[d]	152	21	3			.923	.077		.005[c]
	*Hin*fI	CL/P	109	64	39	6			.766	.234		.229
		Controls	108	52	50	6			.713	.287		
EN2	*Pvu*II	CL/P	76	10	32	34			.342	.658		.511
		Controls	53	11	19	23			.387	.613		
	*Sac*I	CL/P	98	68	27	3			.833	.167		1.000
		Controls	33	22	11	0			.833	.167		
HOX2F	*Msp*I	CL/P	41	18	18	5			.659	.341		.520
		Controls	43	16	20	7			.606	.395		
HOX2G ...	*Taq*I	CL/P	115	0	27	88			.117	.883		.445
		Controls	106	1	18	87			.094	.906		
	*Msp*I	CL/P	71	55	14	2			.873	.127		.411
		Controls	51	42	9	0			.912	.088		
BCL2	*Eco*RI	CL/P	108	33	41	34			.495	.505		1.000
		Controls	80	21	37	22			.494	.506		

[a] Two-tailed.
[b] Frequency of the common allele compared with combined frequencies of rare alleles.
[c] Significant at $P < .05$.
[d] Includes the 63 controls reported by Hayward et al. (1988).

cases for whom all three TGFA RFLPs were genotyped and for 82/110 controls (79% over all, proportions not significantly different), and these are shown in table 3. In the absence of parental data, haplotypes could not be assigned for those who were doubly or trebly heterozygous, but it is assumed that the biases thus created would be similar in cases and controls. There were no significant haplotype-distribution differences in (*a*) male versus female cases, (*b*) all cases versus all controls, (*c*) cases with positive family history versus controls, (*d*) cases with a positive family history versus cases with a negative family history, (*e*) cases with cleft lip and palate versus cases with cleft lip alone, (*f*) cases with cleft lip and palate versus controls, (*g*) cases with cleft lip only versus controls, (*h*) cases with bilateral clefts versus cases with unilateral clefts, (*i*) cases with

Table 3

TGFA Haplotype Data

Haplotype Chromosomes[a]	No. of CL/P Chromosomes	No. of Control Chromosomes
C1A2D1	125	114
C1A1D2	4	4
C1A2D2	26	31
C2A2D1	22	13
C1A1D1	1	1
C2A2D2	1	0
C1A3D1	1	1
Total	180	164

[a] Derived from alleles present at the *Taq*I, *Bam*HI, and *Hin*fI RFLPs in individuals who were heterozygous at no more than one RFLP. The D1 allele is equivalent to the TGFA *Rsa*I B2 allele (Ardinger et al. 1989), and the D2 allele is equivalent to B1.

bilateral clefts versus controls, and (*j*) cases with unilateral clefts versus controls (comparisons *a–g*, $P > .2$; comparisons *h–j*, $P > .1$).

The most common haplotype among cases and controls was C1A2D1, which occurred in 125/180 (70%) of case chromosomes and 114/164 (70%) of control chromosomes. The C2A2D1 haplotype (which is the equivalent of the C2A2B2 referred to by Ardinger et al. [1989] because the D1 *Hin*fI allele is in perfect linkage disequilibrium with the B2 *Rsa*I allele [G. Chenevix-Trench, unpublished data]) occurred in 23/180 (13%) of case chromosomes and 13/164 (8%) of control chromosomes ($P = .16$; two-tail test). Neither was this haplotype significantly more frequent in chromosomes from cases with positive family histories (16/112) than in chromosomes from controls ($P = .11$).

It is of interest to estimate the increased risk of CL/P in individuals carrying the genotypes or alleles that we have found to be significantly associated and to see whether there is any interaction between them. Analysis is based on the joint distribution of TGFA and RARA genotypes, shown in table 4, which excludes Hayward et al.'s (1988) 63 controls for whom no RARA data were available. For homozygous and heterozygous carriers of the TGFA C2 allele (*Taq*I RFLP) the OR (95% confidence interval [CI]) is 2.39 (1.03–5.97). The OR for the RARA A2A2 genotype is 2.08 (1.09–4.00). There is no significant interaction between the TGFA C1 allele and the RARA A2A2 genotype for CL/P risk ($P = .10$; exact test).

It is also of interest to estimate what proportion of total risk for CL/P in this population may be attributed to these two genetic factors. This may also be conceptualized as the proportion of CL/P cases that would not occur if everybody in the population were of genotype TGFA C1C1 and RARA A1A1 or A1A2 (Khoury et al. 1991). We calculated the attributable risk as the $\tilde{\alpha}$ statistic of Thomas and Gart (1992) with Fisher-transformed 95% confidence limits. For TGFA C2 this was .16 (.03–.28), and for RARA A2A2 it was .33 (.10–.53). The estimate of joint attributable risk of these two factors was .47, close to the sum of the individual attributable risks (.49). The larger attributable risk of the RARA polymorphism reflects the high frequency of the A2A2 genotype (.64, vs. only .15 for the C2 allele), despite the larger relative risk associated with the C2 allele.

Yet another way to view these data is to consider the increased risk to a relative of an affected proband that is attributable to their sharing a high-risk allele or genotype. When expressed relative to the population prevalence, this is the recurrence-risk ratio λ_R (Risch 1990). In recent reviews of family studies of clefting, this has been estimated to be in the range 20–40 for first-degree relatives, so a mean of $\lambda_1 = 30$ seems reasonable (Farrall and Holder 1992; Mitchell and Risch 1992). However, on the basis of combined data of Ardinger et al. (1989) and Chenevix-Trench et al. (1991), the recurrence-risk ratio to offspring sharing the TGFA C2 allele with their affected parent is $\lambda_o C_2 = 1.21$, and that for siblings is $\lambda_s C_2 = 1.23$ (Mitchell and Risch 1992); for the current data alone this value is only 1.08, and so it is clear that this polymorphism makes only a modest contribution to the total λ_1. If more than one locus contributes to risk and if the risks attributed to each locus act multiplicatively to produce the total λ_R (Risch 1990; Farrall and Holder 1992), it can be calculated that 16 loci with the same effect size and frequency as this would be needed to account for the empirical recurrence risks. Using the same method of calculation (Risch 1987) for the RARA A2A2 genotype, we find that there is negligible dominance (which is in line with observed greater risk in A2 homozygotes than in heterozygotes), so that $\lambda_{oA2A2} = \lambda_{sA2A2} = 1.06$, an even more modest contribution to recurrence risk; 58 such genes would be needed to account for the observed recurrence risk.

We may also estimate the contributions these genes make to the heritability of clefting, which has been estimated at around .8 in several studies (Farrall and Holder 1992). For our data, the heritabilities due to the TGFA and RARA polymorphisms are each about .01, a negligible contribution to the total genetic variance in liability for clefting.

Table 4

TGFA *Taq*I and RARA *Pst*I Alleles

Genotypes[a]	CL/P Cases	Controls
C1C1 A1A1	5	6
C1C1 A1A2	22	32
C1C1 A2A2	53	27
C1C2 A1A1	2	2
C1C2 A1A2	11[b]	1
C1C2 A2A2	14	6
C2C2 A1A1	0	0
C2C2 A1A2	0	0
C2C2 A2A2	3	1

[a] C1 and C2 are TGFA *Taq*I alleles; and A1 and A2 are RARA *Pst*I alleles.

[b] Includes one C1C2 A2A3 genotype.

Discussion

Our results for the TGFA polymorphisms echo those reported by Ardinger et al. (1989), in some, but not all, respects. The associations of clefting with the *Bam*HI and *Taq*I RFLPs are weaker than those reported by Ardinger et al., and, when the controls typed only for this study were considered, only attain acceptable significance when a one-tail test is used. In our study, the OR (95% CI) for the *Bam*HI A2 allele is 1.78 (0.89–3.50), and that for the *Taq*I C2 allele is 1.77 (1.00–3.26), whereas in Ardinger et al.'s study these values are 3.15 (1.29–7.89) and 2.89 (1.28–6.63), respectively. Only if the controls typed for the *Taq*I RFLP by Hayward et al. (1988) are added to our own does the association with the C2 allele (2.23 [1.30–3.82]) approach the significance reported by Ardinger et al. The *Hin*fI RFLP (equivalent to the *Rsa*I RFLP used by Ardinger et al.) is not significantly associated in either our (1.32 [0.86–2.05]) or Ardinger et al.'s (1.04 [0.64–1.68]) study.

When haplotypes were counted in genotypes in which these could be unambiguously assigned, Ardinger et al. found the C2A2B2 haplotype to be significantly overrepresented in clefting cases (11%), particularly familial ones (17%) versus controls (4%). In our study, we also find the equivalent C2A2D1 haplotype overrepresented among cases (13%), and more so in familial ones (14%), compared with controls (8%), but neither difference is significant, even when one-tail tests are used (P = .10 and .07, respectively). It is likely that much larger numbers of both cases and ethnically matched controls will need to be used if studies are to have consistent findings.

Quite new in our study is the finding of an association of clefting with the A2 allele, detected by the *Pst*I RFLP in the RARA locus. The RARA locus was selected as a candidate gene, in view of the known properties of RA as an environmental teratogen. For this reason it is not clear that the harsh Bonferroni correction of the alpha significance level for the total of seven new RFLPs investigated is entirely warranted (Rothman 1986, pp. 147–150). Nevertheless, the association could well prove to be nothing but a type I error, and only replication in large samples will tell. On the other hand, it might play a role parallel to that of the N-acetyl transferase region of mouse chromosome 8, which appears to govern susceptibility to glucocorticoid-induced cleft palate, as well as to influence susceptibility to other teratogens for clefting in mice (Karolyi et al. 1990). In view of the fact that RA causes cleft palate rather than cleft lip in experimental animal models (Sulik et al. 1988), it is interesting that the association with the RARA RFLP was stronger for CL/P than for cleft lip, and in larger studies this may be a significant effect. There has been a renewed interest in retinoid teratogenicity, since 13-*cis* RA and all-*trans* RA (tretinoin) were adopted as a treatment for severe acne, with resultant malformations in offspring of women who used the treatment while pregnant (Rosa et al. 1986; Camera and Pregliasco 1992). One unifying hypothesis consistent with our findings is that both TGFA and RARA act in response to RA, since it is known that the promoter region of TGFA contains an RA response element (Raja et al. 1992). It is interesting that it has been demonstrated in mice that RA exposure does alter the expression of TGFA (Abbott and Birnbaum 1990). This would suggest a central role for RA, both endogenous and exogenous, in the etiology of both sporadic and familial CL/P.

If the association with the RARA locus does prove to be genuine, its epidemiological importance in the light of our data can be viewed in several different ways. The relative risk associated with the A2 allele is modest (about 2), and its contribution to the heritability of clefting is negligible (about 1%). However, its contribution to the attributable risk may be substantial—about a third of the cases can be attributed to possession of two A2 alleles, and a further 7% can be so attributed if only one is carried. By contrast, the C2 allele detected by the *Taq*I RFLP of the TGFA locus also confers a relative risk of about 2–3 and makes the same negligible contribution to the heritability but would account for substantially less attributable risk (about 12%). It should be noted, however, that the interpretation of attributable risk is problematic when risk factors are interacting (Khoury et al. 1991), as is probably the case with clefting (Farrall and Holder 1992; Mitchell and Risch 1992). In this connection, there is no evidence in our data that the TGFA and RARA loci act other than additively on the logistic scale and hence multiplicatively (i.e., interactively) on the risk scale.Nevertheless, the combined attributable risk of almost 50%, due to these two risk factors, does raise the question of the effect size and frequency of the remaining unfound risk factors and may suggest that these are predominantly of larger effect and lower frequency, with consequent larger recurrence risk to relatives but smaller attributable risk.

One possibility, of course, is that the known RFLPs at the TGFA and RARA loci are in only moderate

linkage disequilibrium with other mutations in these genes, mutations that have much larger effect size. If this is the case, then evidence for linkage should be found in families segregating for clefting. The failure to find evidence of linkage with the TGFA locus (Hecht et al. 1991*a*; Vintiner et al. 1992) is expected in studies of such modest dimensions as theirs, if the risk to relatives is no larger than that revealed by the association studies to date (Risch 1987). The failure also suggests that this locus holds no mutation of larger effect in the families analyzed. Linkage analysis with the RARA locus might be more successful if rarer polymorphisms, with stronger associations with clefting, were first identified.

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