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Linkage Analysis in a Large Brazilian Family with van der Woude Syndrome Suggests the Existence of a Susceptibility Locus for Cleft Palate at 17p11.2-11.1

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Summary

van der Woude syndrome (VWS), which has been mapped to 1q32-41, is characterized by pits and/or sinuses of the lower lip, cleft lip/palate (CL/P), cleft palate (CP), bifid uvula, and hypodontia (H). The expression of VWS, which has incomplete penetrance, is highly variable. Both the occurrence of CL/P and CP within the same genealogy and a recurrence risk <40% for CP among descendants with VWS have suggested that the development of clefts in this syndrome is influenced by modifying genes at other loci. To test this hypothesis, we have conducted linkage analysis in a large Brazilian kindred with VWS, considering as affected the individuals with CP, regardless of whether it is associated with other clinical signs of VWS. Our results suggest that a gene at 17p11.2-11.1, together with the VWS gene at 1p32-41, enhances the probability of CP in an individual carrying the two at-risk genes. If this hypothesis is confirmed in other VWS pedigrees, it will represent one of the first examples of a gene, mapped through linkage analysis, which modifies the expression of a major gene. It will also have important implications for genetic counseling, particularly for more accurately predicting recurrence risks of clefts among the offspring of patients with VWS.

Introduction

van der Woude syndrome (VWS; [MIM 119300]) is a rare autosomal dominant orofacial disorder caused by a gene mapped to 1q32-41, with a penetrance of 89%–90% (Burdick et al. 1985; Murray et al. 1990). The cardinal features of this syndrome are pits and/or sinuses of the lower lip (lip pits [LP]), which are present in ~80% of VWS-gene carriers. These pits are associated with clefts in approximately half of these carriers: two-thirds of those with clefts have cleft lip and/or palate (CL/P), and one-third have cleft palate alone (CP). Hypodontia (H) is present in ~20%–30% of gene carriers and may occasionally represent the sole expression of the gene. Microforms that represent a mild expression of the gene may include a verrucous eminence in the lower lip, submucous CP, and bifid uvula (BU) (Cervenka et al. 1967; Burdick et al. 1985; Schinzel and Klauser 1986). The occurrence of CP and CL/P within the same family or even the same sibship is very intriguing, since these two malformations classically have been regarded as two separate embryological events (Fraser 1970). It has been observed that the risk for CP in a child is significantly higher when the parent has both LP and CL/P than when the parent has LP only (Cervenka et al. 1967; Burdick et al. 1985). An association between the type of cleft in the parent and that in the offspring has also been shown (Shprintzen et al. 1980; Burdick et al. 1985). In order to explain these findings, it has been proposed that individuals who carry the gene for VWS are also subject to any multifactorial influences (environmental and genetic) contributing to clefting. Also, the development of clefts in persons carrying a "lip pit" major gene may depend on the presence of modifying genes at other loci (Cervenka et al. 1967; Shprintzen et al. 1980; Burdick et al. 1985). However, the identification of modifying genes in any genetic disorder requires the analysis of large pedigrees, which are usually very rare, particularly if more than one susceptibility locus is involved.

We have identified a large Brazilian family with VWS in which the phenotypic expression among the patients ranges from complete bilateral CL/P with LP to LP only. One branch of this family, in which 5 of 16 sibs have CP (involving both soft and hard palate), constitutes a

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unique opportunity to search for modifying genes that contribute to the development of CP. In an attempt to achieve this goal, linkage analysis was performed in this particular sibship using a different approach; it counted as affected only those who have CP, regardless of whether it was associated with other clinical alterations of VWS.

Subjects and Methods

Family

This family was ascertained at the Department of Plastic Surgery of the University of São Paulo, Brazil. The pedigree, as far as it could be traced, contains 75 members in four generations: 25 clinically affected individuals and 50 apparently normal ones. Thirty-one DNA samples were obtained for genetic analysis (14 from normal and 17 from affected individuals), and the following individuals were clinically examined: II-3, II-4, III-1, III-3–III-21, and IV-1–IV-8. The clinical classification of the remaining cases was based on information from more than one relative of the family. As indicated in figure 1, the clinical status of I-1 and I-2 was unknown.

Methods

Genotype.—Genomic DNA was extracted from whole blood according to standard techniques (Miller et al. 1988). Linkage to the VWS gene was tested with six polymorphic microsatellite markers from 1q32-41 (D1S245, D1S471, D1S491, D1S205, D1S217, and D1S178; the order of these markers was based on the work of Schutte et al. [1996] and Dib et al. [1996]).

To identify in this genealogy a potential CP susceptibility locus that is not allelic to VWS, we performed a linkage search in the sibship (III-1–III-16), segregating CP as the major clinical sign, independently of the presence of other clinical features of VWS. This criterion was adopted because of our poor understanding of the genetic causes of each of the malformations of this syndrome; it is still unclear whether the malformations have different embryological origins. Because we can not yet distinguish clinically between the dependent and the independent occurrence of CL and CP, all individuals with CL and CP (hard and soft palate) were included as affected. In addition, although it is known that BU may be a consequence of CP, Meskin et al. (1965) showed that in the normal population this malformation may occur in isolation, suggesting that its occurrence may

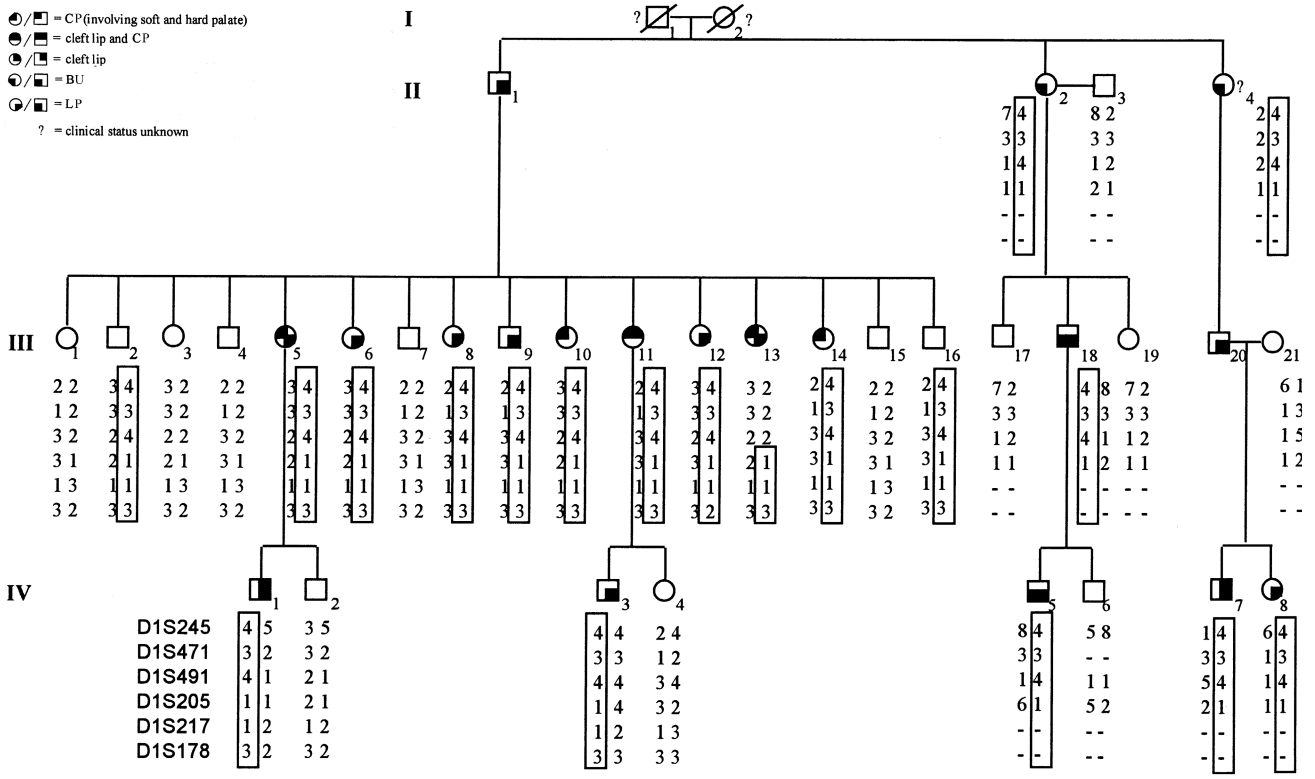

Figure 1 Haplotype analysis for the most informative markers from the 1q32-41 region, that showed linkage to the VWS gene. Only the individuals who were available for DNA studies were included. The at-risk haplotype for VWS is boxed.

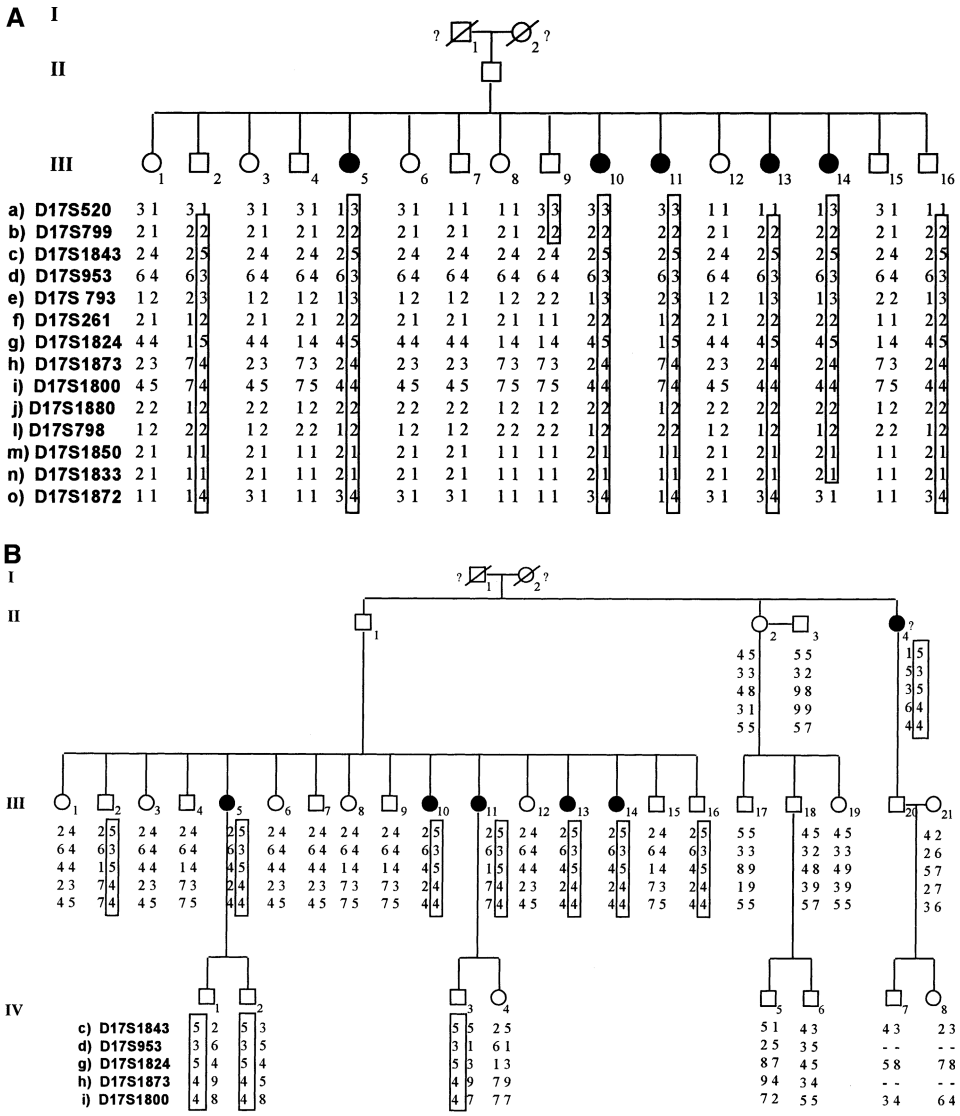

Figure 2 *A*, Haplotype with markers from the 17p11.2-11.1 region in the sibship III-1–III-16. The at-risk haplotype for the CP-predisposing locus in the family is boxed. The order and interlocus distances among the markers are from the Généthon map (Dib et al. 1996). Only individuals with CP, independently of the presence of other clinical features of VWS, are indicated as affected (blackened circles). *B*, Haplotype with the most informative markers from the 17p11.2-11.1 region, in the total family. The at-risk haplotype for the CP-predisposing locus in the family is boxed. Only individuals with CP, independently of the presence of other clinical features of VWS, are indicated as affected (blackened circles).

have a different etiology; therefore, patients who had only BU were not classified as affected. In the sibship selected for linkage analysis, there are seven phenotypically normal individuals and nine patients with at least one clinical feature of VWS: two with CP only, two with CP and LP, one with CL and CP, and four with LP only. For the search of the modifying locus, we considered as affected those five individuals with CP and considered as normal persons the remaining eleven individuals. In a first-stage search, we tested 43 polymorphic markers from genes and/or chromosomal regions that are candidates for the Stickler phenotype (COL2A1, COL11A1, and COL11A2) and for the development of nonsyndromic CL/P and CP, as shown in table 2. In a second screening set, we analyzed 130 microsatellites, spaced ~10–20 cM apart, across 12 autosomes (chromosomes 5 and 12–22). A modification of the PCR-based DNA-pooling strategy was used (Arnheim et al. 1985): 5 individuals considered as affected were analyzed separately and equal amounts of DNA from the 11 sibs classified as normal were used as a DNA control pool. If a shared allele was observed among the patients but at

Table 1

Two-point Linkage Analysis, Between the Most Informative Chromosome 1 Markers and the Disease Gene

Cytogenetic Localization	Locus	LOD Score at θ = 0	.01	.05	.1	.2	.3	.4	Maximum θ	Maximum LOD Score
1q32-41	D1S245	−.32	3.64	4.12	4.05	3.39	2.39	1.14	.06	4.13
1q32-41	D1S471	−1.75	1.68	2.28	2.38	2.07	1.45	.51	.09	2.38
1q32-41	D1S491	−.32	3.64	4.12	4.05	3.39	2.39	1.14	.06	4.13
1q32-41	D1S205	2.36	2.32	2.15	1.94	1.49	1.01	.51	0	2.36

lower intensity than in the DNA control pool, all the individuals from this sibship were then tested for this marker. Evidence of linkage was considered when a LOD score ≥ 1 was observed in this sibship (the Multiple Sclerosis Group 1996). In this case, all the genealogy was tested, and, again, we considered as affected only those individuals with CP, regardless of whether it was associated with other clinical signs of the syndrome (fig. 2). DNA amplification for the individual cases was done with 60 ng of DNA in a 10-μl PCR containing 10 mM Tris-HCl pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl; 0.01% gelatin; 0.1% Triton; 200 μM each of dATP, dTTP, and dGTP; 2.5 mM dCTP; 7.5×10^{-4} μCi of [P32]-dCTP; 4.0 pmol of each primer; and 0.2 U of *Taq* DNA polymerase (HT Biotechnology LTD). The DNA control pool contained 60 ng of DNA from each of the 11 individuals classified as normal, and the PCR conditions were the same as those used for individual cases.

Statistical analysis.—Two-point linkage analysis was performed by use of the MLINK program of the LINKAGE package, version 5.1 (Lathrop et al. 1984). The frequency of the VWS gene, with autosomal dominant inheritance, was assumed to be .001 with a 90% penetrance. For the modifying locus, we also inferred an autosomal dominant inheritance, on the basis of the proposed models of inheritance for clefts (Hecht et al. 1991; Marazita et al. 1992). Two allele frequencies were considered for this putative modifier, .01 and .001, with .50–.90 as the range of penetrance values. Equal allele frequencies were used for all the marker loci; however, allele frequencies for the most informative markers that showed evidence of linkage (D17S1843, D17S953, D17S1824, D17S1873, and D17S1800) were estimated in 80 chromosomes from unrelated healthy Brazilians. Recombination frequencies were assumed to be equal in males and females.

Results

Clinical Characterization of the Genealogy and Linkage Analysis to the VWS Locus

Among the 75 offspring of the ancestral couple, 25 had one or more manifestations of the syndrome: 5 have the classic combination of LP and CL/P, 10 have LP only, 4 have CP only, 2 have bilateral CL/P, 2 have BU and LP, and 2 have only BU.

Linkage analysis with six markers from the 1q32-41 region, performed in 31 members of the genealogy (14 normal individuals and 17 patients with VWS), confirmed linkage to the VWS locus, as detailed in table 1 and summarized in figure 1. Interestingly, we observed two individuals (III-2 and III-16) who have inherited from their affected father the entire at-risk haplotype for VWS but who are themselves phenotypically normal.

Linkage Analysis for the Mapping of a Predisposing Locus for CP in Patients with VWS

Considering now as affected patients only those who have CP, regardless of whether it is associated with other clinical manifestations of VWS (sibs III-1–III-16), no evidence of linkage was observed with any of the 43 microsatellites tested nearby or within 16 candidate genes for nonsyndromic CL/P, CP, and the Stickler phenotype (table 2). Subsequently, we performed a partial genomewide linkage search, using 130 mapped autosomal microsatellite markers in this sibship of interest. No evidence for linkage with any of the other markers was observed, except for the marker D17S1824, for which all five women affected by CP shared an allele that was underrepresented in the control pool. Therefore, all the individual members of this sibship were genotyped. The highest LOD scores (1.92 and 1.95; recombination fraction [θ] 0) were observed with 70% and 80% penetrances, respectively, and, therefore, we considered these values of penetrance for further analysis (table 3). Thirteen additional markers from this 17p11.2-11.1 region were analyzed (D17S520, D17S799, D17S1843, D17S953, D17S793, D17S261, D17S1873, D17S1800, D17S1880, D17S798, D17S1850, D17S1833, and D17S1872). Positive LOD scores were obtained for most of these markers (data not shown), and a common haplotype spanning a 26-cM region was observed in all patients with CP (figure 2*A*). The LOD scores for the most informative markers (D17S1843, D17S953, D17S1824, D17S1873, and D17S1800) are shown in table 3; similar values were obtained independently of the estimated frequency for the modifier locus—that is, .01 or .001.

The most informative markers (D17S1843, D17S953, D17S1824, D17S1873, and D17S1800) were also gen-

Table 2

Candidate Genes and/or Chromosomal Regions for the Development of CL/P and CP, Tested in the Family with VWS

Candidate Gene and/or Chromosomal Region	Location	Markers Used
Growth factors:		
GR1	5q11-q14	D5S806, D5S428, D5S815, D5S107
TGFA	2p13	D2S170, D2S177, D2S405
RARA	17q21.1	D17S250, D17S579
BCL3	19q13.1	D19S47, APOC2
FGFA	5q31.3-33/5q33.3-qter	D5S209, D5S422, D5S400, D5S211
TGFB1	19q13.1	D19S433, D19S224,D19S178
TGFB3	14q23-q24	D14S52, D14S298, D14S53
Homeobox genes:		
HOX2B	17q21-2	HOXB2, D17S809
HOX7 (MSX1)	4q16.1-.3	HOX7, D4S412, D4S394, D4S403
HOX8 (MSX2)	5q35	D5S408
Extracellular matrix proteins:		
COL2A1	12q12-q13.2	D12S59, D12S85
COL9A1	6q12-q14	D6S254, D6S251
COL11A1	1p21	D1S223, D1S206, D1S2896, D1S2671
COL11A2	6p21.2	D6S105, D6S89, D6S276
Chromosomal regions	4q25-4q31.3	D4S175, D4S192
	6p24.3	D6S105, D6S89

otyped in the whole family (fig. 2*B*). For a penetrance of 70%, a peak LOD score of 2.05 at $\theta = 0$ was observed for the marker D17S1824, also suggesting linkage (table 4). Haplotype analysis (fig. 2*B*) showed that five individuals, apparently without CP, have inherited simultaneously the at-risk haplotypes for CP (17p11.2-11.1) as well as for VWS (1q32-41): one has LP associated with CL (IV-1), one has LP only (IV-3), one has severe hypernasal speech and BU (II-4), and two have normal phenotypes (sibs III-2 and III-16). It is also important to notice that the hypernasal speech in patient II-4, who has refused to be examined, can be due to submucous CP.

Discussion

The Mendelian concept of monogenic disease causation appears to be increasingly untenable for a growing number of developmental errors. There are many examples showing that well-delineated clinical entities can be genetically heterogeneous and that distinct mutations in a particular gene may result in fundamental clinical differences. In addition, examples of identical mutations resulting in highly variable phenotypes have been shown for many genetic disorders. However, the underlying mechanisms for these findings are poorly understood. Many hypotheses have been proposed—such as epigenetic genes and interactions among different genes (Wolf 1997). Thein et al. (1994) and Craig et al. (1996) have demonstrated that the heterocellular hereditary persistence of fetal hemoglobin (HPFH) is influenced by several genetic modifiers and have mapped a distinct locus to 6p, unlinked to the β-globin gene complex. This new locus was identified through linkage analysis in a very large kindred of Asian origin. Interestingly, the higher levels of HPFH were segregating with this chromosome region in one branch of the genealogy but not in the other, suggesting the existence of still other modifier loci in the same family. The analysis of this large pedigree demonstrates that the study of single families or even of a single sibship can be very important in the search for susceptibility genes, since such an approach increases the likelihood that genetic homogeneity will be found. In orofacial clefts (OFC), several studies have supported the hypothesis of two or more genes simultaneously involved in their etiology (Farral et al. 1992; Clementi et al. 1995). Recently, Pezzetti et al. (1998) have demonstrated an interactive effect of two disease loci, mapping to 2p13 and to 6p23, that are involved in nonsyndromic OFC malformations in some families.

The Brazilian family with VWS described here is of particular interest, since it has a great number of affected individuals with CP in only one sibship and, therefore, is uniquely well-suited to the search for a modifying (or susceptibility) locus through linkage analysis. The partial genome scan in this sibship showed that all affected patients with CP share a common haplotype both for VWS and for the 17p11.2-11.1 regions. Moreover, if we classify patient II-4 (who has severe hypernasal speech) as affected, the estimated LOD score of 2.05 at $\theta = 0$ for the marker D17S1824 increases to 2.89 at $\theta = 0$ with penetrance of 70%.

If we consider all the individuals with the VWS at-risk haplotype in the present genealogy, and if we include II-4 as affected, the estimated penetrance for CP is 6/19 (31.5%); however, if we consider the presence of both

Table 3

Two-point Linkage Analysis, between Each 17p Most Informative Marker and the Disease Locus, in Sibship III-1–III-16, with Different Penetrance Values

Penetrance and Locus	LOD Score at θ = 0	.01	.05	.1	.2	.3	.4
50%:							
D17S1843	1.68	1.65	1.51	1.34	.96	.56	.18
D17S953	1.68	1.65	1.51	1.34	.96	.56	.18
D17S1824	1.67	1.64	1.51	1.33	.96	.56	.18
D17S1873	1.67	1.64	1.51	1.33	.96	.56	.18
D17S1800	1.67	1.64	1.51	1.33	0.96	.56	.18
60%:							
D17S1843	1.81	1.78	1.65	1.47	1.07	.64	.21
D17S953	1.81	1.78	1.65	1.47	1.07	.64	.21
D17S1824	1.81	1.78	1.64	1.46	1.07	.63	.21
D17S1873	1.81	1.78	1.64	1.46	1.07	.63	.21
D17S1800	1.81	1.78	1.64	1.46	1.07	.63	.21
70%:							
D17S1843	1.92	1.89	1.76	1.60	1.19	.72	.25
D17S953	1.92	1.89	1.76	1.60	1.19	.72	.25
D17S1824	1.92	1.89	1.76	1.59	1.18	.71	.24
D17S1873	1.92	1.89	1.76	1.59	1.18	.71	.24
D17S1800	1.92	1.89	1.76	1.59	1.18	.71	.24
80%:							
D17S1843	1.95	1.93	1.84	1.69	1.30	.82	.29
D17S953	1.95	1.93	1.84	1.69	1.30	.82	.29
D17S1824	1.95	1.93	1.83	1.68	1.29	.81	.28
D17S1873	1.95	1.93	1.83	1.68	1.29	.81	.28
D17S1800	1.95	1.93	1.83	1.68	1.29	.81	.28
90%:							
D17S1843	1.76	1.78	1.79	1.72	1.40	.92	.35
D17S953	1.76	1.78	1.79	1.72	1.40	.92	.35
D17S1824	1.76	1.78	1.79	1.72	1.39	.90	.33
D17S1873	1.76	1.78	1.79	1.72	1.39	.90	.33
D17S1800	1.76	1.78	1.79	1.72	1.39	.90	.33

at-risk haplotypes, the estimated penetrance for CP increases to 6/10 (60%). These observations suggest that the 17p haplotype increases the penetrance of CP in this family, although it does not explain the association of these two malformations in all the patients with VWS. In this regard, it is intriguing that two of five patients without CP who have both "at-risk" haplotypes are also nonpenetrant for the VWS gene. It is also interesting that four of these five nonpenetrant individuals are males, whereas all the other individuals with CP who have both at-risk haplotypes are females. These observations further suggest that other genetic or environmental factors may contribute to the development of the abnormal phenotype. Indeed, the effect of nongenetic factors for the manifestations of clefts in VWS has been illustrated by the observation of a pair of MZ twins with VWS who are discordant for cleft lip but not for LP (Cervenka et al. 1967). Another important point to be addressed is the lack of the 17p at-risk haplotype in the individuals with only BU. However, as reported by Meskin et al. (1965), this condition—the incidence of which is ~1% in whites and ~10% in Native Americans and Japanese Americans, has a frequency of 18% in sibs and parents of affected persons. These observations suggest that this condition is not rare and that it may occur independently of alterations in the palate.

The penetrance for CP in the present genealogy is comparable to the estimates in the literature, which vary from 15% to ~40% among families with VWS (Cervenka et al. 1967; Janku et al. 1980; Burdick et al. 1985). This low penetrance for CP in the great majority of families with VWS raises the possibility that 17p may be a modifying locus that interacts with the 1q gene, increasing the chance that an individual with both at-risk chromosomes will develop cleft palate. Regarding the frequency of this 17p modifier locus, there are two possibilities. The first is that the 17p gene could be one of many modifying loci contributing to CP; in this case, the confirmation of these data in other families with VWS will not be easy. Linkage analysis in many multifactorial diseases, such as schizophrenia, prostate cancer, and nonsyndromic CL/P and CP, have provided good examples of positive findings in one data set that have not been often replicated in other studies (Sherrington et al. 1988; Murray 1995; Eeles et al. 1997; McIndoe et al. 1997). Alternatively, the 17p gene represents a more common modifying locus, and, in this case, the study of other large families with VWS that are segregating CP may confirm our findings. The observation of relatively high (10%–44%) proportions of altered clefts among patients with a deletion at 17p11.2 (Smith et al. 1986; Stratton et al. 1986; Greenberg et al. 1996) is relevant to the present findings, further suggesting the existence, in this region, of a gene predisposing to clefts.

Identifying and characterizing susceptibility or modifying loci is a difficult task, since the degree and type of epistasis, or interaction among them, strongly influ-

Table 4

Two-point Linkage Analysis, between Each 17p Most Informative Marker and the Disease Locus, in All Available Members of the Family, with 70% and 80% Penetrance

Penetrance and Locus	LOD Score at θ = 0	.01	.05	.1	.2	.3	.4
70%:							
D17S1843	1.44	1.44	1.41	1.35	1.12	.77	.32
D17S953	1.10	1.15	1.26	1.28	1.14	.81	.35
D17S1824	2.05	2.04	1.96	1.83	1.48	1.00	.43
D17S1873	1.13	1.14	1.17	1.16	1.02	.72	.30
D17S1800	1.78	1.78	1.73	1.64	1.34	.93	.39
80%:							
D17S1843	1.17	1.19	1.25	1.26	1.13	.81	.35
D17S953	.67	0.79	1.06	1.19	1.16	.87	.39
D17S1824	1.86	1.87	1.88	1.83	1.55	1.10	.49
D17S1873	.61	.67	.84	.96	.96	.74	.33
D17S1800	1.55	1.58	1.63	1.61	1.40	1.00	.45

ence the chance of detecting such genes via a linkage-analysis study. The difficulty is increased further because these genes may vary among families and even among different sibships within one genealogy. We are aware that our findings should be interpreted with caution, since the LOD scores obtained in the present study did not meet the most stringent level of statistical significance for linkage (Lander et al. 1995). However, these results represent the first suggestion of a modifier locus acting together with the VWS gene. The confirmation of a CP-predisposing locus at 17p11.2-11.1, which depends on further studies of other large families with VWS and with multiple cases of CP, will be extremely important for genetic counseling, particularly for more accurately predicting the recurrence risks of clefts among the offspring of patients with VWS.

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Electronic-Database Information

The accession number and URL for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/omim (for VWS [MIM 119300])

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