Published in final edited form as:

Am J Med Genet A. 2006 December 1; 140(23): 2562–2570.

PVRL1 Variants Contribute to Non-Syndromic Cleft Lip and Palate in Multiple Populations

Joseph R. Avila¹, Peter A. Jezewski¹, Alexandre R. Vieira¹, Iêda M. Orioli², Eduardo E. Castilla³, Kaare Christensen⁴, Sandra Daack-Hirsch¹, Paul A. Romitti⁵, and Jeffrey C. Murray^{1,4,5,*}

¹Department of Cytokine Biology, The Forsyth Institute and Department of Developmental Biology, Harvard School of Dental Medicine, Boston, Massachusetts

²Latin American Collaborative Study of Congenital Malformations (ECLAMC) at Department of Genetics, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil

³Latin American Collaborative Study of Congenital Malformations (ECLAMC) at Department of Genetics, Oswaldo Cruz Institute, Rio de Janeiro, RJ, Brazil and CEMIC, Buenos Aires, Argentina

⁴Center for the Prevention of Congenital Malformations, University of Southern Denmark, Denmark

⁵Department of Epidemiology, University of Iowa, Iowa City, Iowa

Abstract

Poliovirus Receptor Like-1 (PVRL1) is a member of the immunoglobulin super family that acts in the initiation and maintenance of epithelial adherens junctions and is mutated in the cleft lip and palate/ectodermal dysplasia 1 syndrome (CLPED1, OMIM #225000). In addition, a common non-sense mutation in *PVRL1* was discovered more often among non-syndromic sporadic clefting cases in Northern Venezuela in a previous case-control study. The present work sought to ascertain the role of PVRL1 in the sporadic forms of orofacial clefting in multiple populations. Multiple rare and common variants from all three splice isoforms were initially ascertained by sequencing 92 Iowan and 86 Filipino cases and CEPH controls. Using a family-based analysis to examine these variants, the common glycine allele of the G361V coding variant was significantly overtransmitted among all orofacial clefting phenotypes ($P = 0.005$). This represented G361V genotyping from over 800 Iowan, Danish, and Filipino families. Among four rare amino acid changes found within the V1 and C1 domains, S112T and T131A were found adjacent to critical amino acid positions within the V1 variable domain, regions previously shown to mediate cell-to-cell and cell-to-virus adhesion. The T131A variant was not found in over 1,300 non-affected control samples although the alanine is found in other species. The serine of the S112T variant position is conserved across all known *PVRL1* sequences. Together these data suggest that both rare and common mutations within *PVRL1* make a minor contribution to disrupting the initiation and regulation of cell-to-cell adhesion and downstream morphogenesis of the embryonic face.

*Correspondence to: Jeffrey C. Murray, 2182 Medical Laboratories, Iowa City, IA 52246. E-mail: jeff-murray@uiowa.edu

Joseph R. Avila and Peter A. Jezewski contributed equally to this work.

Grant sponsor: NIH; Grant numbers: DE08559, DE09160, P50 DE16215, R01 DE11948; Grant sponsor: Fogarty; Grant number: TW 05503.

Peter A. Jezewski's present address is Department of Cytokine Biology, The Forsyth Institute and Department of Developmental Biology, Harvard School of Dental Medicine, Boston, Massachusetts

Keywords

PVRL1 variants; non-syndromic cleft lip; cleft palate

INTRODUCTION

Orofacial clefting results from a disruption of normal embryonic processes. Non-syndromic or isolated cleft lip and/or palate is a birth malformation affecting 1-2 of 1,000 individuals born. These non-syndromic patients account for 70% of births with a cleft while the syndromic forms account for the other 30% divided across over 400 associated forms [Murray, 2002]. Classically, it is thought that clefts that disrupt the primary palate differ from those that disrupt secondary palate formation and hence may result in the two familial patterns of cleft lip and/or palate or cleft palate only (CPO) [Fraser, 1955;Harville et al., 2005]. Recent reports suggest a mixed clefting type is found with mutations in certain genes [Lidral et al., 1998;van den Boogaard et al., 2000;Kondo et al., 2002].

One syndromic form of orofacial clefting is cleft-lip and palate with ectodermal dysplasia, (CLPED, OMIM #225000). CLPED is characterized by cleft lip and palate (CLP) as well as ectodermal abnormalities, such as sparse hair, dysmorphic teeth, and abnormal fingernails. A stop mutation was found in exon 3 of Poliovirus Receptor Like-1 (PVRL1) (W185X) in individuals from Margarita Island. Two frameshift mutations were discovered in *PVRL1* from individuals with the Zlotogora-Ogur (OMIM #225000) form of CLPED. In the Margarita Island ED form of the syndrome, approximately 65% of individuals have an abnormally broad, flat philtrum, cleft lip, or CLP [Bustos et al., 1991;Suzuki et al., 1998]. The W185X obligate heterozygotes also often exhibit a broad, flat upper lip, perhaps a form fruste of CL/P [Bustos et al., 1991;Suzuki et al., 1998;Suzuki et al., 2000]. This broad flat upper lip is reminiscent of the broad flat intermaxillary process found during the 7th week of human development and may represent disrupted morphogenesis downstream of primary palate fusion. This is consistent with loss of downstream signaling from a truncated PVRL1 protein.

PVRL1, also known as Nectin1 [Takahashi et al., 1999], is a receptor-like protein that has an immunoglobulin-like extracellular domain [Bork et al., 1994;Halaby and Mornon, 1998]. There are four known nectins, Nectin1-4, and each has an extracellular, a transmembrane domain, and an intracellular domain [Reymond et al., 2001]. Nectins, such as PVRL1, regulate the velocity of formation of epithelial adherens junctions in cooperation with cadherins, and the subsequent formation of tight junctions on epithelial cells [Honda et al., 2003]. The first step in this process is the formation of homocis dimers between the C1 extracellular domains. This is followed by formation of homo or heterotrans dimers through the interaction of the V domains of other nectin molecules [Yasumi et al., 2003]. PVRL1 has three differently spliced isoforms: α , β , and γ , spliced together from a total 10 exons. Only the α and β isoforms of PVRL1 have an intracellular domain.

PVRL1 also serves as one of the three known primary receptors for alpha herpes virus binding and entry and is known in this context as herpes virus entry mediator C (HveC) [Krummenacher et al., 2003]. Maternal viral infection during pregnancy could influence the risk of cleft lip and/or palate in their children. Higher rates of orofacial clefting have been found in those children whose mothers displayed cold sores during pregnancy, perhaps a marker for viral infection [Romitti et al., 2001].

The above information suggests *PVRL1* may contribute to non-syndromic clefting, as has been shown for other syndromic genes where particular variants contribute to isolated clefting as shown for *TBX22* [Marcano et al., 2004], *MSX1* [Jezewski et al., 2003;Suzuki et al., 2004],

IRF6 [Zuccherro et al., 2004], *FGFR1* [Dode et al., 2003], and others [Vieira et al., 2005]. The present report used direct sequencing of cases and controls to search for mutations in populations from Europe, Asia, and South America. The set of common variants discovered then served as the basis for transmission disequilibrium tests on different case subpopulations with or without infectious exposures during pregnancy.

METHODS

Subjects

All samples were collected with signed consent and had local and/or University of Iowa IRB approval.

Cheek swabs from 147 Danish triads (father, mother, affected child) were collected between October 1999 and March 2000 according to a standard protocol. Subjects included families with at least one case with cleft lip with or without cleft palate (CL/P) and CPO contacted through the University of Southern Denmark.

Samples from South American countries were collected through the Latin-American Collaborative Study of Congenital Malformations (ECLAMC) [Castilla and Lopez-Camello, 1990]. From January 1998 to April 2002, ECLAMC collected blood spots on filter cards from 434 CL/P and CPO patients and their mothers from eight countries in South America: Argentina, Brazil, Bolivia, Chile, Ecuador, Paraguay, Uruguay, and Venezuela. This patient sample was further characterized by subgroups with either prenatal infection exposure or viral exposure. An ECLAMC medical professional compiled the infection exposure information based on indications of fever, flu, pharyngitis, urinary tract infection, sore throat, bronchitis, cold, tuberculosis, toxoplasmosis, vaginal discharge, candidiasis, pneumonia, HIV, sinusitis, human papilloma virus, syphilis, mumps, vaginal infection, Chagas disease, otitis, cold sores, or appendicitis. The viral exposure subgroup data included only fever, flu, cold, sore throat, bronchitis, sinusitis, HIV, mumps, and cold sores.

Cases from the Philippines were studied under the auspices of Operation Smile International [Poe, 1994]. Blood samples were collected from 296 CL/P and CPO patients who were seen and examined at one of four sites within the Philippines (Cavite, Kalibo, Cebu, and Negros).

Blood specimens were collected through the Iowa Child Health Study, a population-based case-control study of orofacial clefts [Romitti et al., 1998]. Samples were from 200 CL/P and CPO available children and their parents, who were born between January 1, 1987 and December 31, 1991 and ascertained by the Iowa Registry For Congenital and Inherited Disorders.

Approximately 170 samples of DNA from unrelated Caucasians were obtained from CEPH (Centre d'Etudes du Polymorphisme Humaine) families [Dausset et al., 1990]. One thousand sixty-four samples of the CEPH Diversity Cell Line Panel samples were also used [Cann et al., 2002].

A summary of the genotyping data by population is presented in Table I.

PCR and Genotyping

DNA was extracted from filter card blood spots using modifications of published protocols [Raskin et al., 1992; Klenerman et al., 1997; Lidral et al., 1997], from cheek swabs [Richards et al., 1993], and whole blood [Oehlmann and Sievertsen, 1995; Miller et al., 1998], according to published protocols. Primer sequences are available at genetics.uiowa.edu/data.html.

Approximately 20 ng of template genomic DNA was analyzed by polymerase chain reaction (PCR). PCR was performed in 1× PCR buffer (10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl) with 200 μM dNTPs and 0.12-0.2 μM primers (final concentration). Thermocycling was carried out with an initial 3 min denaturation at 94°C followed by 40 cycles of: 30 sec at 94°C, 30 sec at 58°C, 30 sec at 72°C, and final extension at 72°C for 3 min.

Nomenclature for the sequence variants at the PVRL1 locus follows those recommendations at the Human Gene Mutation Database (<http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html>). Basically all variant positions are named by reference to the position within the coding portion of the transcript downstream of the start codon. Intronic variants are then named as either downstream or upstream of the closest flanking exon position. In addition, if a reference single nucleotide polymorphism (or refSNP = rs) name was available from the dbSNP database it was also included.

Genotyping was performed for three common SNPs: 73+456C > G/(rs3829260), G361V; 1082G > T/(rs7940667); and 1400-161G > A/(AY445581, variant 696). These polymorphisms were previously known or identified during sequencing reactions described below. The rs3829260 variant position can be found within a CpG island that starts before exon 1 and extends into the intron 1 beyond the end of exon 1 of PVRL1. This SNP is found in 456 nucleotides into this intron 1. The G361V coding variant is found in exon 6 of the beta spliced transcript. The 1400-161G > A variant is found in 161 nucleotides before exon 8 of the beta spliced transcript. Genotyping was carried out on a GeneAmp 7900 Sequence Detection System in 3 μl reaction volumes, in duplicate. PCR reactions were carried out in 1 × TaqMan Universal PCR Master Mix from Applied BioSystems (Foster City, CA), 1 × of the Assay-By-Design primer assay mix, and 2 ng of genomic DNA. PCR was performed with initial enzyme activation at 95°C for 10 min followed by 40 cycles of 92°C for 15 sec and 60°C for 1 min. Genotyping was performed initially in the ECLAMC and Iowa samples. Multiple populations were studied to test whether findings from individual populations could be replicated.

Direct Sequencing

All *PVRL1* exons and exon-intron boundaries were sequenced in both directions. PCR products were used for sequencing reactions directly or after purification by extraction from 2% agarose gels using Qiagen Gel Purification kit (Qiagen, Chatsworth, CA). Cycle sequencing was carried out according to the manufacturer's instructions using ABI PRISM BigDye Terminator Sequencing kits with AmpliTaq DNA polymerase FS (Perkin Elmer, Foster City, CA) on an Applied Biosystems DNA Sequencing System (model 373 or ABI3700 sequencer). Data were collected and analyzed with the Phred/Phrap/Consed System to identify sequence variants [Nickerson et al., 1997].

Protein Sequence Comparisons

Multisequence alignments of *PVRL1* and related sequences were performed with files in FASTA format gathered in Jalview, [Clamp et al., 2004] and submitted for remote alignment at the EBI using a Clustal algorithm. The human PVRL1 sequence was aligned with mouse (Mm 11496257), rat (Rn 27661888), monkey (Ca 11096295), hamster (Ma 11096293), cow (Bt 11096291), lemur (Lc 26106044), and pig (Ss 11096289), PVRL1-related sequences and the PVRL2 human (Hs 13111849) sequence (see Fig. 1 for the V1-domain alignment from these species).

Data Analysis

For linkage disequilibrium the computer program FBAT (family-based association test) was used for Danish, Iowa, and Filipino populations [Horvath et al., 2001]. The likelihood ratio test (LRT) of Weinberg [1999] was used to analyze the ECLAMC population.

RESULTS

DNA Sequencing

Sequence analysis was performed on exons from all splice isoforms of *PVRL1*, as well as the conserved CpG island upstream of the gene, in 92 Iowan and 86 Filipino cases. The intron between exons 5 and 6a of *PVRL1* was also screened for variants that might favor one splice isoform over another, but no variants were found in this region.

DNA sequencing of affected and control populations revealed 23 variants in total. Eleven of these variants were found within coding sequence, while seven were intronic, and another seven were found within CpG islands. Among the coding variants were six mutations that resulted in an amino acid change. Ten polymorphisms were found to have a rare allele frequency greater than 4% among the case and/or control populations. The initial genotype data collected from the Iowan, Filipino, and CEPH population is listed in Table II. Several common variants were found in a GC-rich promoter region at the 5 prime end of *PVRL1*. Other variants were found within exons 1-4, representing the immunoglobulin-like domain. Rare amino acid substitutions included the S112T and T131A variants found within the variable domain and R199Q and R210H found within the constant domain of the immunoglobulin like region. The R199Q variant has been reported previously [Struyf et al., 2002].

As mentioned above, *PVRL1* serves as a binding site for herpes viruses. The minimum alpha-Herpes Simplex Virus entry site on *PVRL1* is found along the C-C' ridge (region I of Fig. 1) of the V-domain [Menotti et al., 2002]. However, regions II and III of *PVRL1* are also important for viral binding and nectin-nectin interactions. These regions represent the E and F beta strands of the protein. The S112T variant, found in a single Filipino proband with CLP, is found within this functionally important E beta strand. The T131A variant, also found in a single Filipino proband with CLP, is found directly adjacent to region III and the F beta strand.

A Taqman assay for the T131A allele was developed to test for its presence within 330 Filipino non-affected controls, 1,027 Filipino cases, and the 1,064 CEPH Diversity Cell Line Panel samples. The only sample that had the T131A variant was the previously identified heterozygote. The presence of the R210H variant was also examined in a similar fashion, in 330 Iowa non-affected controls, 163 Iowa cases, and 1,064 CEPH Diversity Cell line Panel samples. R210H heterozygotes were found in seven controls, two cases, and two CEPH Diversity Panel samples.

A glycine to valine amino acid substitution, G361V, was found within exon 6 of the *PVRL1* beta transcript. This amino acid is within the transmembrane domain of the protein. Glycine at this position is apparently the ancestral amino acid since it is found at this position in chimps, dogs, mice, and rats. The valine rare allele has a frequency of approximately 12% in Iowa cleft cases, 6% in Filipino cleft cases, and 10% in CEPH controls.

The online ESEfinder examines whether a single nucleotide polymorphisms could alter exon splice enhancer (ESE) sites. The G361V; 1082G > T variant produces a significant ESE site score change indicating an ESE site is present with the T allele and is completely absent with the G allele [Cartegni et al., 2003].

Linkage Disequilibrium

Family-based transmission disequilibrium tests were used to examine potential associations between common alleles at the *PVRL1* locus and orofacial clefting phenotypes: cleft lip only (CLO), CLP, CPO, both cleft lip and CLP (all lip), and both CPO and cleft lip and palate (all palate). These genotype analyses were run on 205 nuclear families (615 persons) from Iowa, 307 nuclear families (921 persons) from the Philippines, 298 nuclear families (1,180 persons) from Denmark, and 322 families (517 persons) from ECLAMC (see Table I). Table III presents the results.

The ECLAMC cleft palate population was associated with the 1400-161G > A marker ($P = 0.04$) with a large number of informative samples, although this result was not replicated in the All Palate group.

The Iowa CLO population was associated with the G361V coding variant ($P = 0.04$) but this was with only four informative families. The G361V variant demonstrated a trend towards association with all clefts in the Iowa, Danish, and Filipino populations. To examine this possibility further, the G361V data from the Iowa, Danish, and Filipino populations was pooled together, representing genotyping from more than 800 families. This combination resulted in a more significant association between all types of clefting and the G361V variant ($P = 0.005$). There was also a trend towards significant association with either all lip or all palate when these three populations were summed.

Since ECLAMC patient samples had prenatal infection or viral exposure data, these probands were divided into subgroups for analysis as shown in Table IV. These data were analyzed using the LRT of Weinberg to allow for missing parents in this sample set.

For the ECLAMC G361V data set, no significant associations were found for any of the markers whether prenatal signs of viral or other infection existed. The ECLAMC virally exposed CLO probands with a “viral exposure” demonstrated mild association ($P = 0.0414$) with the 1400-161G > A marker.

DISCUSSION

The present work sought to follow-up the reports that demonstrate a role for *PVRL1* in the CLPED syndrome. This locus was examined in multiple populations that exhibited non-syndromic forms of orofacial clefting. Variants at this locus were initially ascertained by sequencing case and control populations. Three common variants from the *PVRL1* locus were evaluated using family-based approaches. In addition, those samples from South America that had prenatal infection data were evaluated for a possible gene-environment interaction.

Analysis of *PVRL1* Variants Derived by Sequencing

The coding, intronic and promoter regions of all *PVRL1* spliced isoforms were sequenced in Iowan and Filipino cases and CEPH control populations. The sequencing work revealed a set of rare and common variants at this locus.

Among the rare mutations discovered, the mutations found in the second and third exon are within the V1 and C1 extracellular domain of *PVRL1*. These variants could negatively influence *PVRL1* protein function by interfering with dimerization of *PVRL1*/nectin molecules during epithelial adhesion events. The R199Q and R210H variants are found within the constant domain that mediates the formation of homo-*cis*-dimers and thus may disrupt their formation. The complete conservation of the arginine found at these variant sites supports their significance.

The S112T and T131A V-domain variants could influence protein function by interfering with proper cell-to-cell adhesion and by altering viral interactions. Nectin3 and Nectin4 are PVRL1-related proteins that bind to the predicted C-C' -C'' -D beta strands of the *PVRL1* V1-variable domain [Fabre et al., 2002]. However, others have suggested that the EF beta strands are also important for such transinteractions between epithelial cells [Miyahara et al., 2000]. The S112T and T131A variants are found within the highly conserved E and F beta strands. This portion of *PVRL1* is made up of a sequence block conserved across all PVRL1s, which includes the functionally important regions II and III. Mutations in region II within the E beta strand, caused the intracellular accumulation of PVRL1 and had a deleterious effect on all activities studied, including virus-nectin interaction, nectin-nectin interaction, and gD-nectin interaction [Struyf et al., 2002].

The threonine at position 131 is changed to the hydrophobic alanine in one proband. This T131A variant is found just past the F beta strand within one amino acid of region III. This location influences viral binding or homotypic transbinding [Struyf et al., 2002]. Thus, it is not surprising that mutations in region III reduce homotypic transinteraction of Nectin2 and reduce entry of other viruses (PRVirus and BHV-1).

Nectin becomes associated with the E-cadherin complex through their respective cytoplasmic tail binding proteins, afadin and catenin [Katata et al., 2003]. Previously reported mutations have been shown to truncate both alpha and beta isoforms of the protein [Suzuki et al., 2000]. The loss of the intracellular domain may disrupt the protein-protein interactions necessary to activate the cell-cell adhesion process. Intracellular variants could disrupt downstream signaling from this domain and influence morphogenesis subsequent to facial prominence fusion.

The W185X non-sense mutation reported as a susceptibility allele in clefting in Northern Venezuela was not found in any of the sequenced non-syndromic cleft populations [Sozen et al., 2001]. The 146 CL/P patients and 100 unaffected individuals of an Italian population also did not show the W185X mutation in any samples [Scapoli et al., 2004].

Linkage Disequilibrium

The FBAT results provide evidence of association between clefting and the *PVRL1* markers. The G361V variant that demonstrated the most consistent associations with all types of clefting ($P = 0.005$) could either be a disease marker or could be a potential disease mutation. This is suggested by the predicted ESE score change.

The minor association between all forms of clefts in these three populations and the G361V marker represents the only *PVRL1* genotype result that was consistent across several populations and cleft types. However, there was little to no evidence for this variant having a role in the South American population. Note that the syndromic CLPED has clefts of the lip and palate. The association data from this report suggests that *PVRL1* mutations influence the likelihood of clefts of both the lip and palate among non-syndromic orofacial clefting families. This data set does not allow conclusions to be drawn about the susceptibility to CPO forms of clefting.

Effects of Viral Infections on Clefting

Previous results have suggested that a maternal viral infection during the prenatal period could adversely affect the frequency of clefting in the child. For example, pregnant mothers with a viral infection, as judged by the presence of a cold sore, have a higher chance of a child being born with CL/P [Romitti et al., 2001]. However, no association between prenatal viral or other

infection during pregnancy and clefts in the ECLAMC data was found. Supporting this finding is the report of retrospective interview data on probable hyperthermia during the critical period of orofacial clefts development in 992 mothers of boys and girls with various clefts from the Czech Republic. These children showed no significant associations between the incidence of maternal febrile illness and various orofacial subgroups in the children [Peterka et al., 1994]. Future studies should focus specifically on herpes virus infection to test the potential association between clefts and this viral infection.

In summary, *PVRL1* missense mutations may be rare causes of orofacial clefts. One of these, G361V, has a significantly overtransmitted allele in multiple populations. Measures of the interactions of *PVRL1* with other genes and their proteins may also provide more power with large samples to detect interaction effects. Similarly, accurate characterization of maternal first trimester viral infection may also provide better power to the study of gene by environment interactions.

ACKNOWLEDGMENTS

We are especially grateful to the many patients and their families who willingly participated in these studies. Many laboratory personnel also provided much needed assistance with multiple aspects of this study including but not limited to Astanand Jugessur, Min Shi, Anne Warrington, and Theresa Zuccherro.

REFERENCES

- Bork P, Holm L, Sander C. The immunoglobulin fold. Structural classification, sequence patterns and common core. *J Mol Biol* 1994;242:309–320. [PubMed: 7932691]
- Bustos T, Simosa V, Pinto-Cisternas J, Abramovits W, Jolay L, Rodriguez L, Fernandez L, Ramela M. Autosomal recessive ectodermal dysplasia. I. An undescribed dysplasia/malformation syndrome. *Am J Med Genet* 1991;41:398–404. [PubMed: 1776626]
- Cann HM, de Toma C, Cazes L, Legrand MF, Morel V, Piouffre L, Bodmer J, Bodmer WF, Bonne-Tamir B, Cambon-Thomsen A, Chen Z, Chu J, Carcassi C, Contu L, Du R, Excoffier L, Ferrara GB, Friedlaender JS, Groot H, Gurwitz D, Jenkins T, Herrera RJ, Huang X, Kidd J, Kidd KK, Langaney A, Lin AA, Mehdi SQ, Parham P, Piazza A, Pistillo MP, Qian Y, Shu Q, Xu J, Zhu S, Weber JL, Greely HT, Feldman MW, Thomas G, Dausset J, Cavalli-Sforza LL. A human genome diversity cell line panel. *Science* 2002;296:261–262. [PubMed: 11954565]
- Cartegni L, Wang J, Zhu Z, Zhang MQ, Krainer AR. ESEfinder: A web resource to identify exonic splicing enhancers. *Nucleic Acid Res* 2003;31:3568–3571. [PubMed: 12824367]
- Castilla, E.; Lopez-Camello, J. The surveillance of birth defects in South America. I. The search for time clusters: Epidemics.. In: Obe, G., editor. *Advances in mutagenesis research*. Springer-Verlag; Berlin: 1990. p. 191-210.
- Clamp M, Cuff J, Searle SM, Barton GJ. The Jalview Java alignment editor. *Bioinformatics* 2004;20:426–427. [PubMed: 14960472]
- Dausset J, Cann H, Cohen D, Lathrop M, Lalouel JM, White R. Centre d'étude du polymorphisme humain (CEPH): Collaborative genetic mapping of the human genome. *Genomics* 1990;6:575–577. [PubMed: 2184120]
- Dode C, Levilliers J, Dupont JM, De Paepe A, Le Du N, Soussi-Yanicostas N, Coimbra RS, Delmaghani S, Compain-Nouaille S, Baverel F, Pecheux C, Le Tessier D, Cruaud C, Delpech M, Speleman F, Vermeulen S, Amalfitano A, Bachelot Y, Bouchard P, Cabrol S, Carel JC, Delemarre-van de Waal H, Goulet-Salmon B, Kottler ML, Richard O, Sanchez-Franco F, Saura R, Young J, Petit C, Hardelin JP. Loss-of-function mutations in *FGFR1* cause autosomal dominant Kallmann syndrome. *Nat Genet* 2003;33:463–465. [PubMed: 12627230]
- Fabre S, Reymond N, Cocchi F, Menotti L, Dubreuil P, Campadelli-Fiume G, Lopez M. Prominent role of the Ig-like V domain in trans-interactions of nectins. Nectin3 and Nectin4 bind to the predicted C-C'-D beta-strands of the nectin1 V domain. *J Biol Chem* 2002;277:27006–27013. [PubMed: 12011057]

- Fraser F. Thoughts on the etiology of clefts of the palate and lip. *Acta Genet Stat Med* 1955;5:358–369. [PubMed: 13339079]
- Halaby DM, Mornon JP. The immunoglobulin superfamily: An insight on its tissular, species, and functional diversity. *J Mol Evol* 1998;46:389–400. [PubMed: 9541533]
- Harville EW, Wilcox AJ, Lie RT, Vindenes H, Abyholm F. Cleft lip and palate versus cleft lip only: Are they distinct defects? *Am J Epidemiol* 2005;162:448–453. [PubMed: 16076837]
- Honda T, Shimizu K, Fukuhara A, Irie K, Takai Y. Regulation by nectin of the velocity of the formation of adherens junctions and tight junctions. *Biochem Biophys Res Commun* 2003;306:104–109. [PubMed: 12788073]
- Horvath S, Xu X, Laird N. The family based association test method: Strategies for studying general genotype-phenotype associations. *Eur J Hum Genet* 2001;9:301–306. [PubMed: 11313775]
- Jezewski PA, Vieira AR, Nishimura C, Ludwig B, Johnson M, O'Brien SE, Daack-Hirsch S, Schultz RE, Weber A, Nepomucena B, Romitti PA, Christensen K, Orioli IM, Castilla EE, Machida J, Natsume N, Murray JC. Complete sequencing shows a role for MSX1 in non-syndromic cleft lip and palate. *J Med Genet* 2003;40:399–407. [PubMed: 12807959]
- Katata T, Irie K, Fukuhara A, Kawakatsu T, Yamada A, Shimizu K, Takai Y. Involvement of nectin in the localization of IQGAP1 at the cell-cell adhesion sites through the actin cytoskeleton in Madin-Darby canine kidney cells. *Oncogene* 2003;22:2097–2109. [PubMed: 12687012]
- Klenerman P, Henzgartner H, Zinkernagel RM. A non-retroviral RNA virus persists in DNA form. *Nature* 1997;390:298. [PubMed: 9384383]
- Kondo S, Schutte BC, Richardson RJ, Bjork BC, Knight AS, Watanabe Y, Howard E, de Lima RL, Daack-Hirsch S, Sander A, McDonald-McGinn DM, Zackai EH, Lammer EJ, Aylsworth AS, Ardinger HH, Lidral AC, Pober BR, Moreno L, Arcos-Burgos M, Valencia C, Houdayer C, Bahuaui M, Moretti-Ferreira D, Richieri-Costa A, Dixon MJ, Murray JC. Mutations in IRF6 cause Van der Woude and popliteal pterygium syndromes. *Nat Genet* 2002;32:285–289. [PubMed: 12219090]
- Krummenacher C, Baribaud I, Eisenberg RJ, Cohen GH. Cellular localization of nectin-1 and glycoprotein D during herpes simplex virus infection. *J Virol* 2003;77:8985–8989. [PubMed: 12885915]
- Lidral AC, Murray JC, Buetow KH, Basart AM, Scheerer H, Shiang R, Naval A, Layda E, Magee K, Magee W. Studies of the candidate genes TGFB2, MSX1, TGFA, and TGFB3 in the etiology of cleft lip and palate in the Philippines. *Cleft Palate Craniofac J* 1997;34:1–6. [PubMed: 9003904]
- Lidral AC, Romitti PA, Basart AM, Doetschman T, Leysens NJ, Daack-Hirsch S, Semina EV, Johnson LR, Machida J, Burds A, Parnell TJ, Rubenstein JL, Murray JC. Association of MSX1 and TGF3 with nonsyndromic clefting in humans. *Am J Hum Genet* 1998;63:557–568. [PubMed: 9683588]
- Marciano AC, Doudney K, Braybrook C, Squires R, Patton MA, Lees MM, Richieri-Costa A, Lidral AC, Murray JC, Moore GE, Stanier P. TBX22 mutations are a frequent cause of cleft palate. *J Med Genet* 2004;41:68–74. [PubMed: 14729838]
- Menotti L, Cocchi F, Campadelli-Fiume G. Critical residues in the CC' ridge of the human nectin1 receptor V domain enable herpes simplex virus entry into the cell and act synergistically with the downstream region. *Virology* 2002;301:6–12. [PubMed: 12359441]
- Miller S, Dykes D, Polesky H. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1998;16:1215. [PubMed: 3344216]
- Miyahara M, Nakanishi H, Takahashi K, Satoh-Horikawa K, Tachibana K, Takai Y. Interaction of nectin with afadin is necessary for its clustering at cell-cell contact sites but not for its cis dimerization or trans interaction. *J Biol Chem* 2000;275:613–618. [PubMed: 10617658]
- Murray JC. Gene/environment causes of cleft lip and/or palate. *Clin Genet* 2002;61:248–256. [PubMed: 12030886]
- Nickerson D, Tobe V, Taylor S. Polyphred: Automating the detection and genotyping of single nucleotide substitutions using fluorescence based resequencing. *Nucleic Acids Res* 1997;25:2745–2751. [PubMed: 9207020]
- Oehlmann R, Sievertsen J. Chaga's disease: In search of a susceptibility gene. *Qiagen News* 1995;3:9–11.

- Peterka M, Tvrdek M, Likovsky Z, Peterkova R, Fara M. Maternal hyperthermia and infection as one of possible causes of orofacial clefts. *Acta Chir Plast* 1994;36:114–118. [PubMed: 7610756]
- Poe D. Operation Smile International: Missions of mercy. *Plast Surg Nurs* 1994;14:225–230. [PubMed: 7732103]
- Raskin S, Phillips JA III, Kaplan G, McClure M, Vnencak-Jones C. Cystic fibrosis genotyping by direct PCR analysis of Guthrie blood spots. *PCR Methods Appl* 1992;2:154–156. [PubMed: 1362128]
- Reymond N, Fabre S, Lecocq E, Adelaide J, Dubreuil P, Lopez M. Nectin4/PRR4, a new afadin-associated member of the nectin family that trans-interacts with nectin1/PRR1 through V domain interaction. *J Biol Chem* 2001;276:43205–43215. [PubMed: 11544254]
- Richards B, Skoletsky J, Shuber AP, Balfour R, Stern RC, Dorkin HL, Parad RB, Witt D, Klinger KW. Multiplex PCR amplification from the CFTR gene using DNA prepared from buccal brushes/swabs. *Hum Mol Genet* 1993;2:159–163. [PubMed: 7684637]
- Romitti PA, Munger RG, Murray JC, Daack-Hirsch S, Hanson JW, Burns TL. The effect of follow-up on limiting non-participation bias in genetic epidemiologic investigations. *Eur J Epidemiol* 1998;14:129–138. [PubMed: 9556171]
- Romitti PA, Murray JC, Daack-Hirsh S, Burns TL. Cold sores and orofacial clefts. *Teratology* 2001;63:252.
- Scapoli L, Palmieri A, Pezzetti F, Carinci F, Marchesini J, Martinelli M, Delaiti G, Tognon M, Carinci P, Gombos F. Investigation of the W185X nonsense mutation of PVRL1 gene in Italian nonsyndromic cleft lip and palate patients. *Am J Med Genet Part A* 2004;127A:211.
- Sozen MA, Suzuki K, Tolarova MM, Bustos T, Fernandez Iglesias JE, Spritz RA. Mutation of PVRL1 is associated with sporadic, non-syndromic cleft lip/palate in Northern Venezuela. *Nat Genet* 2001;29:141–142. [PubMed: 11559849]
- Struyf F, Martinez WM, Spear PG. Mutations in the N-terminal domains of nectin-1 and nectin-2 reveal differences in requirements for entry of various alphaherpesviruses and for nectin-nectin interactions. *J Virol* 2002;76:12940–12950. [PubMed: 12438620]
- Suzuki K, Bustos T, Spritz RA. Linkage disequilibrium mapping of the gene for Margarita Island ectodermal dysplasia (ED4) to 11q23. *Am J Hum Genet* 1998;63:1102–1107. [PubMed: 9758630]
- Suzuki K, Hu D, Bustos T, Zlotogora J, Richieri-Costa A, Helms JA, Spritz RA. Mutations of PVRL1, encoding a cell-cell adhesion molecule/herpesvirus receptor, in cleft lip/palateectodermal dysplasia. *Nat Genet* 2000;25:427–430. [PubMed: 10932188]
- Suzuki Y, Jezewski PA, Machida J, Watanabe Y, Shi M, Cooper ME, Viet le T, Nguyen TD, Hai H, Natsume N, Shimozato K, Marazita ML, Murray JC. In a Vietnamese population, MSX1 variants contribute to cleft lip and palate. *Genet Med* 2004;6:117–125. [PubMed: 15354328]
- Takahashi K, Nakanishi H, Miyahara M, Mandai K, Satoh K, Satoh A, Nishioka H, Aoki J, Nomoto A, Mizoguchi A, Takai Y. Nectin/PRR: An immunoglobulin-like cell adhesion molecule recruited to cadherin-based adherens junctions through interaction with Afadin, a PDZ domain-containing protein. *J Cell Biol* 1999;145:539–549. [PubMed: 10225955]
- van den Boogaard MJ, Dorland M, Beemer FA, van Amstel HK. MSX1 mutation is associated with orofacial clefting and tooth agenesis in humans. *Nat Genet* 2000;24:342–343. [PubMed: 10742093]
- Vieira AR, Avila JR, Daack-Hirsch S, Dragan E, Felix TM, Rahimov F, Harrington J, Schultz RR, Watanabe Y, Johnson M, Fang J, O'Brien SE, Orioli IM, Castilla EE, Fitzpatrick DR, Jiang R, Marazita ML, Murray JC. Medical sequencing of candidate genes for nonsyndromic cleft lip and palate. *PLoS Genet* 2005;1:e64. [PubMed: 16327884]
- Weinberg CR. Allowing for missing parents in genetic studies of case-parent triads. *Am J Hum Genet* 1999;64:1186–1193. [PubMed: 10090904]
- Yasumi M, Shimizu K, Honda T, Takeuchi M, Takai Y. Role of each immunoglobulin-like loop of nectin for its cell-cell adhesion activity. *Biochem Biophys Res Commun* 2003;302:61–66. [PubMed: 12593848]
- Zuccherro TM, Cooper ME, Maher BS, Daack-Hirsch S, Nepomuceno B, Ribeiro L, Caprau D, Christensen K, Suzuki Y, Machida J, Natsume N, Yoshiura K, Vieira AR, Orioli IM, Castilla EE, Moreno L, Arcos-Burgos M, Lidral AC, Field LL, Liu YE, Ray A, Goldstein TH, Schultz RE, Shi M, Johnson MK, Kondo S, Schutte BC, Marazita ML, Murray JC. Interferon regulatory factor 6

(IRF6) gene variants and the risk of isolated cleft lip or palate. N Engl J Med 2004;351:769–780.
[PubMed: 15317890]

V Domain:

	A	A'	B	C	C'	C''
HsN1	HSQVVQVND	SMYGF	IGTDVVLHCSFANPLPSVKITQVTWQKS	TNGSKQNVAIYNPSMGVS		
CaN1			-----S-----	ITQAT-----V-----S-----		
LcN1			-----A-----S-----	A-----T-----T-----V-----A-----		
MmN1	-T-----		-----S-----	A-----S-----M-----T-----		
SsN1	-T-----		-----G-----	A-----T-----V-----A-----		
BtN1			-----G-----	A-----T-----V-----A-----		
HsN2	QDVR---	LPEVR-QL-GT-E-P-HLLP-V-GLYISL---	RPDAPA-	H----	AFH-K----	
						
				I		
	C''	D	E	F	G	
HsN1	VLAP	YRERVEFLRPSFT	DGTIRLSRLELEDEGVYICEFATFPTGNRESQLNLTVM			
CaN1	--A-	-RE-----T	-----SR-----V-----T-----			
LcN1	--A-	-RE-----T	-----SH-----V-----T-----			
MmN1	--P-	-EK-----I	-----SG-----M-----T-----			
SsN1	--A-	-RE-----T	-----SR-----V-----A-----			
BtN1	--A-	-RE-----T	-----SR-----V-----A-----			
HsN2	FPS-KPGSERLSFVSAKQSTGQDTEAELQ-A-LA-HG-TV----	N-T-----	K-SVRGMTW-R-I			
			...	...		
			II	III		

C1 Domain:

HsN1	DKVLVATCT	SANGKPPSVVSWETRLKGEAEYQEIRNPN	GTVTVISRYRLVPSREAHQQSLACIVNYHMD
CaN1	-----	-----R-----	-----R-----
MmN1	-----	-----A-----	-----R-----
RnN1	-----	-----R-----	-----R-----
MaN1	-----	-----R-----	-----R-----
SsN1	-----	-----H-----	-----R-----
BtN1	E-----	-----R-----	-----R-----
HsN2	-PTT-L-I-KE-R-ARI-LSS-DW-KETQVSGTLA----	T-RFT----	GR-DGVTVT-K-EH

FIG 1.

Multisequence alignment of representative PVRL1/Nectin1 V1 and C1 domains with human Nectin2. The putative beta strands of PVRL1 V1-domain are indicated by the overbars. The beta strands labeled as A through G make up the two beta sheets of the V1 immunoglobulin domain. Sites that are 100% conserved across all sequences are indicated by dashes (-), gaps by underscores (_). Bolded residues within the Nectin1 sequences are 100% conserved with the positions shown as dashes below, while those conserved between Nectin1 and Nectin2 are also dashed. The sites of the amino acid variants reported in this document are indicated in bold red in all conserved species. The red letters correspond to amino acid variants S112T and T131A in the V-domain and amino acid variants R199Q and R210H in the C1-domain, respectively. N1 represents Nectin1; N2 represents Nectin2. Hs=Homo sapiens, Le=Lemur catta, Ca=Cercopithecus aethiops (African green monkey), Mm=Mus Musculus, Rn=Rattus Norvegicus, Ma=Mesocricetus auratus, Ss Sus scrofa, Bt=Bos Taurus. Regions=I, II, and III, indicated by "•" are sites of mutations affecting viral binding or homotypic transbinding [Struyf et al., 2002]. ▼=Represents the position of the point mutation, F136L, discovered during subcloning of Nectin2 alpha fragments, which prevented the transinteraction and cell-to-cell aggregation [Miyahara et al., 2000]. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I

Case and Control Subject Totals by Population

Population description	Iowa	ECLAMC	Philippines	Danish	CEPH(controls)
Number of families	205	391 (only mother and proband)	307	298	170
Number of individuals	615	434	921	1,181	170
Number of individuals sequenced	96	—	96	—	170
Number of individuals geno-typed	615	394	921	1,181	1,064

ECLAMC, refers to Estudio Colaborativo Latino Americano de Malformaciones Congénitas or the Latin American Collaborative Study of Congenital Malformations.
CEPH, Centre d'étude du polymorphisme humain, Paris, France.

TABLE II
Initial PVRL1 Genotype Data From the Iowan, Filipino, and CEPH Populations

Region ^a	Exon	Allele	Iowa	Philippines	CEPH	OFC/FHX ^b
CpG/Intron 1		(rs3829260); 79 + 456C >G	27 C/C, 38 C/G, 17 G/G	24 G/G, 41 G/C, 17 C/C	19 C/C, 21 C/G, 12 G/G	
V1	2	S112T; 334T >A	81 T/T	86 T/T; 1 T/A	150 T/T	CLP/?
V1	2	T131A; 391A >G	81 A/A	82 A/A, 1 A/G	150 A/A	CLP/
C1	3	R199Q; 596G >A	91 G/G, 1 G/A, 1 A/A	91 G/G	102 G/G	CLP/+
C1	3	R210H; 629G >A	91 G/G, 2 G/A	91 G/G	102 G/G	CL/+
IC	6a	(rs3833765); 437insE; 1480insGAG	54 +/+, 8 +/ins	78 +/+, 7 +/ins	71 +/+, 10 +/ins, 1 ins/ins	
TM	6b	(rs7940667); G361V; 1082G >T	57 G/G, 13 G/T, 2 T/T	80 G/G, 10 G/T	70 G/G, 15 G/T, 1 T/T	
<8b		(AY445581, variant 696); 1400-161G >A	32 G/G, 38 G/A, 15 A/A	44 G/G, 17 G/A, 4 A/A	29 G/G, 29 G/A, 11 A/A	

^aThe region of the protein where the variant is found is as indicated: CpG, CpG island; V1, variable domain 1; C1, constant domain 1; IC, intracellular domain; TM, transmembrane domain; <8b, intron prior to exon 8.

^bOFC is the orofacial clefting clinical type. This is followed by the family history of orofacial clefting in that case family.

TABLE III

Family-Based Association Test Results for Case Populations by Cleft Type

Variant name ^d and population genotyped	FBA T ^b results by cleft type				
	All clefts	CLO	CLP	CPO	All lip
79 + 456C >G					
Iowa	0.1394	0.3937	1	0.0880	0.5862
ECLAMC	0.7838	0.3448	0.2873	0.8093	0.6889
G361V; 1082G >T					
Iowa	0.0704	0.0455	0.8185	0.4053	0.2971
Danish	0.0929	0.4431	0.3937	0.3657	0.2514
Filipinos	0.1797	1	0.1779	—	0.2857
ECLAMC	0.9056	0.1352	0.2523	1	1
Iowa + Danish + Filipinos	0.0052	0.2396	0.1456	0.2206	0.0614
1400-161G >A					
Iowa	0.7388	0.5637	0.3034	0.8272	0.5553
ECLAMC	0.6630	0.8548	0.6616	0.0416	0.8163

CLO, cleft lip only; CLP, cleft lip and/or palate; CPO, cleft palate only.

^a Variants are named following the recommendations at the Human Gene Mutation Database (<http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html>). In addition, if a reference single nucleotide polymorphism (or refSNP = rs) name was available from the dbSNP database it was also included.

^b FBAT, family-based association test.

TABLE IV
Likelihood Ratio Test Results in the South American Population Among Probands With Either any Prenatal Total Infection or any Viral Infection

Variant name and population genotyped	Infection status	LRT results by cleft type				
		All clefts	CLO	CLP	CPO	All lip All palate
ECLAMC	Total infection	79 + 456C > G				
	Yes	0.8946	0.1895	0.3699	0.2501	0.7729
	No	0.7502	0.4679	0.5519	0.9942	0.8448
	Viral infection					
ECLAMC	Yes	0.7649	0.5482	0.9514	1	0.7901
	No	0.5651	0.4271	0.9514	1	0.5138
	Total infection	G361V; 1082G > T				
	Yes	0.7671	1	0.6083	0.6274	0.6747
ECLAMC	No	0.8509	0.0959	0.2504	1	0.6982
	Viral infection					
	Yes	0.3579	1	0.7171	1	0.3579
	No	0.7692	0.0534	0.2613	1	0.6457
ECLAMC	Total infection	1400-161G > A				
	Yes	0.8906	0.9707	0.657	0.1891	0.7385
	No	0.6244	0.7994	0.8475	0.0931	0.9894
	Viral infection					
	Yes	0.792	0.0414	0.7165	0.1751	0.5254
	No	0.7202	0.3985	0.516	0.1064	0.9916