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CRISPLD2 VARIANTS INCLUDING A C471T SILENT MUTATION MAY CONTRIBUTE TO NONSYNDROMIC CLEFT LIP WITH OR WITHOUT CLEFT PALATE

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Abstract

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Objective—To assess association between nonsyndromic (NS) cleft lip with or without cleft palate (CL(P)) and SNPs within the *CRISPLD2* gene (cysteine-rich secretory protein LCCL domain containing 2).

Design—Four SNPs within the *CRISPLD2* gene domain (rs1546124, rs8061351, rs2326398, rs4783099) were genotyped to test for association via family-based association methods.

Participants—5,826 individuals from 1,331 families in which one or more family member is affected with CL(P).

Results—Evidence of association was seen for SNP rs1546124 in USA (p=0.02) and Brazilian (p=0.04) Caucasian cohorts. We also found association of SNP rs1546124 with cleft palate alone (CP) in South Americans (Guatemala and ECLAMC) and combined Hispanics (Guatemala, ECLAMC and Texas Hispanics) (p=0.03 for both comparisons), and with both cleft lip with cleft palate (CLP; p=0.04) and CL(P) (p=0.02) in North Americans. Strong evidence of association was found for SNP rs2326398 with CP in Asian populations (p=0.003) and with CL(P) in Hispanics (p=0.03), and also with bilateral CL(P) in the Brazilians (p=0.004). In the Brazilians, SNP rs8061351 showed association with cleft subgroups incomplete CL(P) (p=0.004), and unilateral incomplete CL(P) (p=0.003). Prediction of SNP functionality revealed that the C allele in the C471T silent mutation (overrepresented in cases with CL(P) presents two putative exonic splicing enhancer motifs and creates a binding site AP-2 alpha, a transcription factor involved in craniofacial development.

Conclusions—Our results support the hypothesis that variants in the *CRISPLD2* gene may be involved in the etiology of NS CL(P).

Keywords

CRISPLD2 gene; cleft lip; cleft palate; subphenotypes

INTRODUCTION

Craniofacial anomalies, and in particular oral-facial clefts including cleft lip (CL) and cleft palate (CP), are major human structural birth defects with a worldwide frequency of 1 in 700 live births and substantial clinical impact. The possible etiologies are many, including single-gene disorders, chromosome aberrations, exposure to teratogens, and sporadic conditions of unknown cause (Murray, 2002). Oral-facial clefts can be further classified as nonsyndromic (NS, i.e. isolated) or syndromic based on the presence of other structural anomalies. Approximately 30% of all clefts are associated with one of more than 400 described syndromes (Gorlin et al., 2001) while the remaining 70% are isolated defects. It is generally accepted that CL with or without CP (CL(P)) and cleft palate alone (CP) are developmentally distinct phenotypes. CL(P) is more common, affecting 1–2/1000 births and presenting considerable differences in prevalence, with Native Americans and Asians showing the highest rate and Africans the lowest. CP is less common, with a prevalence of approximately 1/1500–2000 births in Caucasians, less variable among different ethnic backgrounds (Forrester and Merz, 2004).

The nature of the genetic contribution to the etiology of NS CL(P) and CP is still a subject for discussion and investigation. Analyses of familial recurrence risk patterns in CL(P) have estimated that 3–14 genes interacting multiplicatively may be involved, indicating that CL(P) is a heterogeneous disorder (Schliekelman and Slatkin, 2002). Nevertheless, despite the evidence for a genetic role in the etiology of CL(P) and CP, environmental factors such as smoking and maternal nutrition are also thought to influence this structural birth defect, possibly in an interactive manner (Maestri et al., 1997; Wyszynski et al., 1997). Therefore,

etiological heterogeneity has probably been a major confounding factor for identifying clefting susceptibility loci.

Recent advances in high-throughput genotyping technologies and powerful statistical approaches have accelerated the discovery of loci conferring susceptibility for complex diseases through the use of genome scans (Altmuller et al., 2001). The first CL(P) scan was conducted with 92 British sib pairs and identified nine regions with suggestive results, including a region on chromosome 16q (Prescott et al., 2000; Prescott et al., 2001). Other genomic scans followed, and the region of 16q21-24 reached genome-wide statistical significance for linkage with CL(P) in multiple studies (Field et al., 2004; Marazita et al., 2004a; Marazita et al., 2004b; Marazita et al., 2009).

CRISPLD2 (cysteine-rich secretory protein containing LCCL domain 2) gene is located on chromosome 16q24.1 and has been recently associated with nonsyndromic CL(P) in U.S. Caucasian and Hispanic populations (Chiquet et al., 2007). Moreover, the authors detected *CRISPLD2* expression in the mandible, palate and nasopharynx regions during craniofacial development at E13.5-E17.5, and have suggested *CRISPLD2* as a novel candidate gene for the etiology of NS CL(P). Although the function of *CRISPLD2* remains to be elucidated, its structure featuring the presence of a LCCL (Limulus factor C, Coch-5b2 and Lgl1) domain has been suggested to play a structural or immunologic role, or even be involved in cell motility (Liepinsh et al., 2001; Nagai et al., 2007). Interestingly, cell motility is required for effective cell migration which, together with apoptosis, accounts for the cellular mechanism responsible for the disappearance of medial edge epithelia cells prior to palatal fusion (Chai and Maxson, 2006).

Given the observed population differences in CL(P) and CP birth prevalences and other characteristics, it is of interest to expand studies of orofacial cleft etiology to diverse populations. In this study, we performed association studies with *CRISPLD2* and NS CL(P) and NS CP in a large cohort sample consisting of distinct populations from North America, South America, Asia, Northern and Eastern Europe.

SUBJECTS AND METHODS

Subjects

The study population consisted of a total of 5,826 genotyped individuals from distinct population sets. Included were 1,023 multiplex families from the Philippines, Guatemala, Europe (Spain, Hungary, Turkey) and the USA (Iowa, Pennsylvania, Missouri, and Texas). In addition, there were 308 nuclear trios, 84 from China plus 224 from Argentina, Brazil and Chile ascertained through ECLAMC (Latin American Collaborative Study of Congenital Malformations). There were also 610 unrelated individuals (328 NS CL(P) cases and 282 controls with no known family history of clefting) of Caucasian ethnicity from Brazil. Table 1 summarizes all of the populations, families and individuals. With the exception of ECLAMC which is a hospital-based birth defects registry study, all families were ascertained by recruitment, either from community-based ascertainment (the Philippines) or through clinical-facility-based ascertainment (remainder of sites).

All cases had NS CL, CP or CL with CP (CLP). CL and CLP combined comprise CL(P). Informed consent was obtained from each study subject after approval by the appropriate institutional review boards in the USA (University of Pittsburgh, Pittsburgh, PA; University of Iowa, IA; Washington University, St. Louis, MO; University of Texas, Houston, TX) and each participating international site (details provided on request).

Blood or saliva samples were collected to obtain genomic DNA. Procedures for DNA extraction were performed according to standard protocols.

Genotyping

Four single nucleotide polymorphisms (SNPs) within the *CRISPLD2* gene domain previously shown to be associated with CL(P) (Chiquet et al., 2007) were genotyped using Taqman chemistry on an automatic sequence-detection instrument (ABI Prism 7900HT, Applied Biosystems, Foster City, CA). Assays and reagents were supplied by Applied Biosystems (Applied Biosystems, Foster City, CA). Details of the studied polymorphisms are presented in Table 2. Further, Table 3 presents the allelic and genotypic frequencies by population subgroups, plus the results of tests of Hardy Weinberg Equilibrium (HWE).

Statistical Analysis

Families were analyzed separately according to population and then in subgroups. We combined all the North American Caucasian samples from Pittsburgh, Saint Louis, Iowa and Texas to form a North American group. The European Caucasians (Turkey, Spain and Hungary) were added to the North American group to form an overall Caucasian group. The data from Guatemala and ECLAMC formed the South American group. Note that the Guatemalans and the registry participants from ECLAMC are of European (Spain and Portugal) extractions as well as a mixture of Amer-Indians and Africans. A Hispanic group was formed with the self-reported Hispanics from Texas and the South American group, to address any inquiries as to ethnic environmental considerations.

For some analyses the families were divided into the following non-overlapping subsets: CL=families in which all affecteds had CL only; CLP=all affecteds had CL plus CP; CLCLP=at least one affected with CL plus at least one with CLP; CP=at least one affected with CP alone. Another grouping was designated CL(P) and consisted of the combination of the CL, CLP and CLCLP subsets.

The properties of the SNPs were assessed in the proband trios using Haploview (Barrett et al., 2005), ie., to assess LD between the SNPs, to test for Hardy-Weinberg Equilibrium (HWE), and to estimate the minor allele frequencies (MAF).

The association between clefting and SNPs in the *CRISPLD2* gene was determined by Transmission Disequilibrium Tests (TDT) in the family data, as implemented in FBAT (Family Based Association Test, version 1.7.3) (Laird et al., 2000; Rabinowitz and Laird, 2000; Horvath et al., 2001). Allelic, genotypic, and haplotype TDT analyses were performed with the empirical analysis option in order to adjust for families with multiple parent-child trios. Parent of origin effects were assessed in the proband trios using PLINK (Purcell et al., 2007). For the Brazilian case-control sample, allelic/genotypic associations were assessed with chi-square tests as implemented in SAS (version 9.1.3).

Nominal p-values are reported. With a Bonferroni correction for multiple testing (considering the number of variables and tests performed), p-values below 0.0006 (0.05/80) would be considered statistically significant.

RESULTS

Preliminary analyses

Pairwise linkage disequilibrium (D’ values) between the four SNPs were calculated for each population using the GOLD (Graphical Overview of Linkage Disequilibrium) program (Abecasis and Cookson, 2000) and ranged from 0.02 to 0.23; thus it is likely that very little

redundant information was obtained from the data (data not shown). Table 3 presents the allelic and genotypic frequencies, and the p-values from the tests of HWE by each subpopulation. There was no evidence of deviation from Hardy-Weinberg equilibrium for any of the SNP/population group combinations, except for SNP rs4783099 in the North American Caucasian and ALL Caucasian groups.

Association analyses

The results of case-control and family-based allelic association analyses stratified by population are summarized in Table 4. None of these results reached formal Bonferroni-adjusted significance (i.e. p-value < 0.0006), however results in 2 SNPs (rs1546124 and rs8061351) were nominally significant ($p<0.05$) for the Brazilian case-control sample (Caucasian), the USA Caucasians, and suggestive ($0.5\leq p<0.10$) in Turkey; in addition to the original results from Texas (Chiquet et al., 2007). Interestingly, the results from Asia and the Latin American admixed populations (Guatemala and ECLAMC) were not significant for any SNP.

Table 5 shows the results from TDT allelic association analyses by cleft family subgroups. There are some suggestions in the results that the CLP subgroup (i.e. families in which all affecteds have CL plus CP) have the greatest statistical significance in the Caucasian populations for SNP rs1546124. Of interest, in the Asian subgroup there was a suggestive result in the CP family subgroup ($p =0.003$) however the sample size was very small (15 informative families out of 37 total CP families). The haplotype TDT association results are not presented in detail because in no case did the significance levels improve over the individual SNP results. Similarly, there was no significant evidence of parent-of-origin effects for any SNP, therefore those results are not presented in detail.

Table 6 presents the Brazilian case-control results for both allelic and genotypic association for all cases and for subgroups of the cases based on cleft laterality and completeness (Letra et al., 2007). Again, no formal significance was seen, but results for three of the SNPs (rs1546124, rs8061351 and rs2326398) were nominally significant or suggestive. The most significant results were with SNP rs8061351 for Incomplete clefts (allelic p-value=0.009, genotypic 0.004), and for Unilateral Incomplete clefts (genotypic p-value=0.003); and with SNP rs2326398 for Bilateral (allelic p-value=0.004) and Bilateral Complete (allelic p-value=0.007).

Prediction of SNP Functionality

In order to verify if the synonymous mutation C471T (rs8061351) in exon 4 of the *CRISPLD2* gene associated with incomplete cleft lip/palate in the Brazilian individuals and in the Hispanic families from Texas (Table 4) could disrupt DNA-binding sites and further affect *CRISPLD2* protein expression, two transcription binding site prediction methods, FASTSNP and AliBaba 2.1, were used (Matys et al., 2006;Yuan et al., 2006). FASTSNP identified that the C allele, overrepresented in cases with cleft lip/palate, affects splicing regulation by altering exonic splicing enhancer motifs; FASTSNP attributes a low-medium risk for this allele. Further, AliBaba 2.1 identified a Sp1 binding site with either C or T alleles, however the C allele also harbored a binding site for transcription factor AP-2 alpha.

DISCUSSION

The current study sought to replicate the recent study (Chiquet et al., 2007) that identified the *CRISPLD2* (cysteine-rich secretory protein containing LCCL domain 2) gene as a novel candidate gene for NS CL(P). *CRISPLD2* is located on chromosome 16q24.1, spans approximately 8.95kb, and contains 14 exons coding for a 497 amino acid polypeptide. The

exact function of this gene is not yet known, nevertheless i*n situ* hybridization of mouse tissues showed *CRISPLD2* expression in the naso- and oropharynx at E13.5, the mandible at E14.5, and the palate and cartilage primordia of the nasal septum at E17.5. (Chiquet et al., 2007).

We investigated the SNPs associated in the original study (Chiquet et al., 2007) in a large NS CL(P) and CP cohort consisting of twelve distinct populations (see Table 1). Although not reaching formal Bonferroni-adjusted significance, several results were suggestive for CL(P) in the Caucasian populations but interestingly not in the Asian nor admixed populations, while the Asian populations had suggestive results for CP. Of note, statistical significance was increased when cases were stratified based on cleft phenotypes.

One polymorphism (rs1546124), located at the 5′ UTR of the *CRISPLD2* gene, showed significant altered transmission in the Caucasian cohort in the original study (Chiquet et al., 2007) and was the only SNP significantly associated with CL(P) in that study. In the current study, a synonymous mutation in exon 4 (C471T, rs8061351) showed association with the case-control cohort from Brazil, and was also shown to be associated with the Hispanic cohort in the original study (Chiquet et al., 2007). Furthermore, our results also suggest that SNP rs1546124 may have a stronger effect on individuals of Caucasian ethnicity whereas SNP rs8061351 seems to have a stronger effect on individuals of Hispanic or South American origin. A possible explanation for this discrepancy is that distinct populations may have different risk alleles.

Recently a study of 31 SNPs in 12 candidate genes including *CRISPLD2* was conducted in an Irish study, comprising 509 CL(P) and 383 CP case-trios, and 926 population-based-controls, and including three of the four SNPs analyzed in the current study (Carter et al., 2010). This study population is most comparable to the ALL Caucasian subgroup in the current study. Notably, for the three SNPs in common between the studies (rs1546124, rs8061351, rs4783099) the minor alleles (G, C, T respectively) and MAF values in the current study (see Table 3) were comparable to those presented in Table 2 of the Irish study (Carter et al., 2010). However, the Irish study found no statistically significant association with any of the three SNPs in common, nor with three additional *CRISPLD2* SNPs assessed only in the Irish study. One of the SNPs in common, rs1546124 showed suggestive association in the current study Caucasians. These differing results could be in part because the study designs differed. Our study population was enriched in multiplex families since most were ascertained as part of a linkage study (Marazita et al., 2009), whereas 7.5% of the Irish study cases had a positive family history of orofacial clefts. Notably, the region of 16q21-24 reached genome-wide statistical significance for linkage with CL(P) in multiple studies (Field et al., 2004; Marazita et al., 2004a; Marazita et al., 2004b; Marazita et al., 2009), consistent with an hypothesis that the *CRISPLD2* relationship with CL(P) may be most important in familial cases.

Of note, several chromosomal abnormalities involving chromosome 16 have been described that include clefting as part of the clinical phenotype. Duplications of 16p12-13 (Brewer et al., 1999), as well as trisomy and translocations of chromosome 16 (Ducos et al., 2004) have been associated with cleft palate. In addition, one marker at approximately 7 kb from the *MMP25* gene on chromosome 16p13.3 has shown association with NS CL(P) (Blanton et al., 2004). More specifically, the region between 16q21-24 has been identified in genomic scans as harboring susceptibility genes for CL(P) (Prescott et al., 2000; Field et al., 2004; Marazita et al., 2004a; Marazita et al., 2004b; Marazita et al., 2009). Further, other investigators have also demonstrated the presence of CP with mutations in genes located in or around 16q21-24. Hecht et al (Hecht et al., 1991) observed a correlation between cleft

palate and variations at 16q22.1, while mutations in the *FOXC2* gene located at 16q24.3 have been shown to cause distichiasis, lymphedema, and CP (Bahuau et al., 2002).

Although the function of *CRISPLD2* is unknown, effects of mutations in its structure may be predicted. It has been proposed that SNP rs1546124, a C/G polymorphism located at the 5' UTR, may have a functional effect on *CRISPLD2* transcription by the presence of a Sp1 binding site whenever a G allele is present (Nagai et al., 2007). We further verified that SNP rs8061351, which denotes a C471T silent mutation with both nucleotides coding for a proline, also implies differences in functionality depending on the allele present. We observed that the C allele, which is overrepresented in the cases with clefts of the lip and of the primary palate, alters splicing regulation by diminishing exonic splicing enhancer motifs. Moreover, the C allele also creates a binding site for transcription factor AP-2 alpha, involved in craniofacial development and recently suggested as an important regulatory element for *IRF6* expression and in turn, for the occurrence of CL(P). Nevertheless, although synonymous changes should not affect gene expression nor the final protein product, they may lead to the synthesis of a protein product with the same amino acid sequence but different structural and functional properties and thus should not be neglected in determining the risk of development of various diseases (Komar, 2007b; Komar, 2007a).

Whether variants in the *CRISPLD2* gene are causal agents for CL(P) is yet to be confirmed. So far, the only biological evidence for a possible role in palate development is the expression of *CRISPLD2* at E17.5 in mice, a time point that does not seem to be critical since the process of palatogenesis is known to be complete by E15 (Dudas et al., 2007). Nonetheless, the associations observed in this and other genetic studies (Blanton et al., 2004; Chiquet et al., 2007; Marazita et al., 2009), and the observations on SNP functionality warrant further investigations to clarify the role for *CRISPLD2* in NS CL(P) and CP suggested by our results..

CONCLUSIONS

This study further demonstrates that variants in the *CRISPLD2* gene may be involved in the pathogenic mechanism of NS CL(P), utilizing a much larger sample size and additional ethnicities beyond the original publication (Chiquet et al., 2007). Nevertheless, the exact biological functions and the contribution of *CRISPLD2* to the clefting phenotype are still to be clarified.

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Table 1

Summary of participants by population and phenotypic subgroups.

		FAMILIES						NUMBER OF INDIVIDUALS GENOTYPED		
				Number of families in phenotype subgroups[a]						
Population Group	**Country or State (USA)**	**Number**	**Mean family size ± s.d.**	**CL**	**CLP**	**CLCLP**	**CP**	**Affected**	**Unaffected**	**Unknown**
Asian	China	84	3.0 ± 0.0	13	47	n/a	24	84	168	0
	Philippines	204	13.4 ± 8.3	15	72	104	13	564	1127	13
Central Am.	Guatemala	95	6.11 ± 3.9	15	70	7	3	80	304	0
South Am.	ECLAMC	224	3.0 ± 0.0	43	132	n/a	49	209	423	0
	Brazil [b]	n/a	n/a	n/a	n/a	n/a	n/a	328	282	0
Europe	Spain	39	3.8 ± 0.94	5	24	4	6	49	97	0
	Hungary	31	5.0 ± 0.98	6	8	10	7	52	71	0
	Turkey	40	9.7 ± 2.7	7	15	7	11	39	66	0
USA	IA	140	3.0 ± 0.0	39	56	3	42	124	222	33
	PA + MO	130	5.3 ± 2.6	14	45	42	29	137	304	0
	TX- Cauc [c]	262	4.8 ± 3.8	45	191	24	2	335	506	1
	TX- Hispanic [c]	82	5.12 ± 3.7	8	71	2	1	87	131	0
TOTAL		**1,331**	**5.8 ± 5.3**	**211**	**731**	**203**	**187**	**2,088**	**3,701**	**47**

[a] Phenotype subgroups: CL=those pedigrees in which all affected members have CL only; CLP = affected members all have CL with CP; CLCLP= at least one affected member with CL and one with CLP; CP=at least one affected member with CP. NOTE: the analysis group CL(P)= CL + CLP + CLCLP.

[b] Brazilian study subjects were unrelated cases (CL=4; CLP=324), and controls (i.e. not families).

[c] Families originally reported (Chiquet et al., 2007)

Table 2

Summary of the SNPs studied in the *CRISPLD2* gene.

SNP Marker	Base Position[a]	Region	Base Change[b]	Average Heterozygozity[a]	Type of Assay
rs1546124	83,429,542	5′ UTR	C/G	0.473 +/− 0.113	Taqman OD[c]
rs8061351	83,440,593	Exon 4	C/T[d]	0.493 +/− 0.059	Taqman OD
rs2326398	83,460,208	Intron 8	A/G	0.499 +/− 0.022	Taqman OD
rs4783099	83,473,468	3′ UTR	C/T	0.471 +/− 0.117	Taqman OD

[a] According to the USCS Genome Browser Human 2004 May Assembly.

[b] Ancestral allele listed first.

[c] Assay-on-demand.

[d] Silent mutation.

Table 3

CRISPLD2 SNPs: HardyWeinberg Equilibrium (HWE), allele and genotype frequencies as calculated from the proband trios.

	SNP MINOR ALLELE		SNP GENOTYPE FREQUENCY			
Population[a]/SNP	**Minor Allele**	**MinorAllele Frequency (MAF)**	**Minor Allele Homozygote**	**Heterozygote**	**Common Allele Homozygote**	**HWE p-value**
South/Central American						
rs1546124	G	29%	10%	37%	53%	0.15
rs8061351	ng	ng	ng	ng	ng	ng
rs2326398	G	22%	5%	36%	59%	0.29
rs4783099	ng	ng	ng	ng	ng	ng
Hispanic						
rs1546124	G	30%	11%	38%	51%	0.10
rs8061351	C	33%	11%	43%	46%	0.84
rs2326398	G	24%	6%	36%	58%	1.0
rs4783099	T	43%	16%	54%	30%	0.38
ALL Caucasian						
rs1546124	G	32%	11%	41%	48%	0.12
rs8061351	C	28%	8%	39%	53%	0.62
rs2326398	G	33%	10%	45%	45%	0.63
rs4783099	T	36%	11%	50%	39%	0.0006
North American Caucasian						
rs1546124	G	32%	11%	41%	48%	0.14
rs8061351	C	28%	8%	39%	53%	0.49
rs2326398	G	32%	11%	43%	46%	0.72
rs4783099	T	37%	11%	50%	39%	0.006
Asian						
rs1546124	C	47%	21%	51%	28%	0.70
rs8061351	C	38%	16%	44%	40%	0.25
rs2326398	A	26%	7%	38%	55%	0.81
rs4783099	C	42%	18%	50%	33%	0.75

[a] Population subgroups (see Table 1): **South/Central American**=Guatemala and ECLAMC; **Hispanic**=Guatamala, ECLAMC, Texas-Hispanic; **ALL Caucasian**=Iowa, Texas-Caucasian, Pittsburgh and St Louis, Madrid, Hungary, Turkey; **North American Caucasian**=Iowa, Texas-Caucasian, Pittsburgh and St Louis;**Asian**= Philippines, China

[b] ng = not genotyped

Table 4

Association of *CRISPLD2* and NS CL(P): Results of chi-square analysis on total Brazilian case-control sample and TDT analysis in families from multiple populations

	Case- Control (χ^2 p- value)	p-values from family-based TDT for CL(P)[a]									
		UNITED STATES									
	BRAZIL	Texas[b]		Total USA[c]	ASIA		EUROPE			CENTRAL AMERICA	SOUTH AMERICA
Ethnicity	**Cauc.**	**Cauc**	**Hisp**	**Cauc**	**Phil**	**China**	**Spain**	**Hungary**	**Turkey**	**Guatemala**	**ECLAMC[d]**
Number of Families	**n/a**	**262**	**82**	**532**	**204**	**84**	**39**	**31**	**40**	**95**	**224**
SNP:											
rs1546124	***0.04***	***0.01***	0.53	***0.02***	0.37	0.27	0.87	n/a	**0.09**	0.98	0.30
rs8061351	***0.04***	0.76	***0.02***	0.85	0.40	0.59	0.27	0.85	0.76	0.43	---
rs2326398	0.11	0.19	**0.06**	0.20	0.24	0.16	0.83	n/a	0.10	0.19	0.43
rs4783099	1.00	0.17	0.22	0.54	0.80	0.90	0.41	0.68	0.13	0.89	---

n/a = not applicable because not enough informative families; Cauc=Caucasian ethinicity, Hisp=Hispanic, Phil=the Philippines

[a] p-values from FBAT analysis of families, values in ***bold italics*** reach nominal significance (ie p-value<0.05), values in **bold** are suggestive (0.05<p-value<0.10).

[b] Results from original *CRISPLD2* report (Chiquet et al., 2007)

[c] "Total USA Cauc" includes Pittsburgh, St. Louis, Iowa and Texas-Caucasian

[d] ECLAMC families were not genotyped for rs8061351 and rs4783099

Table 5

Results from TDT allelic association analyses within cleft family subgroups and population subgroups for SNPs rs1546124 and rs2326398

		CLEFT FAMILY SUBGROUP [a]									
		CL		CLP		CLCLP		CL(P)		CP	
POPULATION [b]	SNP	p-value (allele) [c]	N [d]	p-value (allele)	N	p-value (allele)	N	p-value (allele)	N	p-value (allele)	N
South/Central America	rs1546124	0.56 (G)	32	0.23 (C)	71	NA	4	0.43 (C)	107	***0.03*** (C)	23
	rs2326398	**0.07 (A)**	17	0.66 (A)	61	NA	1	0.17 (A)	79	0.67 (A)	16
Hispanic	rs1546124	0.56 (G)	32	0.13 (C)	85	NA	4	0.26 (C)	121	***0.03*** (C)	23
	rs2326398	**0.09** (A)	19	0.21 (A)	79	NA	2	***0.03*** (A)	100	0.67 (A)	16
North American Caucasian	rs1546124	0.88 (C)	33	***0.04*** (C)	70	0.13 (C)	32	***0.02*** (C)	135	0.59 (C)	24
	rs2326398	0.89 (G)	43	0.12 (A)	96	0.75 (A)	31	0.20 (A)	170	0.81 (G)	23
ALL Caucasian	rs1546124	0.67 (G)	39	0.11 (C)	95	0.36 (C)	42	0.13 (C)	176	0.38 (C)	26
	rs2326398	0.54 (G)	54	**0.10** (A)	122	0.65 (A)	40	0.22 (A)	216	0.74 (A)	34
Asian	rs1546124	0.16 (G)	18	0.35 (C)	61	0.78 (C)	60	0.72 (C)	139	0.81 (G)	20
	rs2326398	0.83 (G)	16	1.00 (G)	64	0.49 (G)	60	0.57 (G)	140	***0.003*** (G)	15

[a] Cleft Family subgroups : CL=those pedigrees in which all affected members have CL only; CLP = affected members all have CL with CP; CLCLP= at least one affected member with CL and one with CLP; CL(P)= cleft with or without cleft palate familes; i.e. a combination of the CL, CLP, CLCLP subgroups; CP=at least one affected member with CP.

[b] Population subgroups: **South/Central America**=Guatemala and ECLAMC; **Hispanic**=Guatamala, ECLAMC, Texas-Hispanic; **North American Caucasian**=Iowa, Texas-Caucasian, Pittsburgh and St Louis; **ALL Caucasian**=Iowa, Texas-Caucasian, Pittsburgh and St Louis, Madrid, Hungary, Turkey; **Asian**= Philippines, China

[c] p-value (allele) = TDT p-value and associated allele. P-values in ***bold italics*** reach nominal significance (ie p-value<0.05), values in **bold** are suggestive (0.05<p-value<0.10).

[d] n: the number of informative families in the TDT (see Table 1 for the number of families in each cleft family subgroup)

Table 6

Brazilian case-control results for allelic and genotypic association with CL(P) subphenotypes (Letra et al., 2007).

			P-VALUES FROM CHI-SQUARE ANALYSES OF EACH *CRISPLD2* SNP [a]							
			rs1546124		rs8061351		rs2326398		rs4783099	
Phenotype	**cases**	**controls**	**Allelic**	**Genotypic**	**Allelic**	**Genotypic**	**Allelic**	**Genotypic**	**Allelic**	**Genotypic**
Subgroup [a]	**N [b]**	**N**	**(C, 70%)[c]**	**(C/C, 49%)**	**(C, 40%)**	**(C/C, 18%)**	**(A, 73%)**	**(A/A, 55%)**	**n/a**	**n/a**
TOTAL	328	282	***0.04***	**0.07**	***0.04***	**0.03**	0.11	0.27	1.00	1.00
Unilateral	157	n/a	***0.02***	**0.05**	0.14	**0.07**	0.96	0.88	0.80	0.63
Bilateral	125	n/a	0.54	0.83	***0.04***	**0.05**	***0.004***	***0.02***	0.29	0.47
Complete	86	n/a	0.12	0.33	0.20	0.19	0.14	0.24	1.00	0.97
Incomplete	196	n/a	**0.09**	**0.07**	***0.009***	***0.004***	0.34	0.30	0.35	0.38
Uni+Comp	64	n/a	**0.05**	0.17	0.64	0.82	0.69	0.22	0.46	0.73
Uni+InComp	93	n/a	**0.08**	0.12	***0.04***	***0.003***	0.67	0.40	0.62	0.39
Bi+Comp	22	n/a	0.62	0.86	0.12	**0.08**	***0.007***	***0.03***	0.47	0.60
Bi+InComp	103	n/a	0.64	0.43	**0.05**	0.13	0.20	0.43	0.25	0.53

[a] p-values from case-control analyses, values in ***bold italics*** reach nominal significance (ie p-value<0.05), values in **bold** are suggestive (0.05<p-value<0.10).

[b] Phenotype Subgroups: groupings of the cleft cases, TOTAL = all cases, Unilateral and Bilateral refer to Laterality, Complete and Incomplete refer to the completeness of the cleft; Uni+Comp=unilateral and complete; Uni+InComp=Unilateral and Incomplete, Bi+Comp=bilateral and complete; Bi+InComp=bilateral and incomplete

[c] N=number or cases or controls, note that some of the phenotype subgroup numbers do not add up to the TOTAL because some of the cases did not have laterality or completeness information

[d] in parentheses is the associated allele or genotype for those SNPs in which one or more p-value < 0.10