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Nonsyndromic cleft lip and palate: CRISPLD Genes and the Folate Gene Pathway Connection

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Abstract

Nonsyndromic cleft lip with or without cleft palate (NSCLP) is a common birth defect that has a multifactorial etiology. Despite having substantial genetic liability, less than 15% of the genetic contribution to NSCLP has been delineated. In our efforts to dissect the genetics of NSCLP, we found that variation in the CRISPLD2 (cysteine rich secretory protein LCCL domain containing 2) gene is associated with NSCLP and that the protein is expressed in the developing murine craniofacies. In addition, we found suggestive linkage of NSCLP (LOD>1.0) to the chromosomal region on 8q13.2-21.13 that contains the CRISPLD1 gene. The protein products of both CRISPLD1 and CRISPLD2 contain more cysteine residues than comparably sized proteins. Interestingly, the folic acid pathway produces endogenous cysteines, and variation in genes in this pathway is associated with NSCLP. Based on these observations, we hypothesized that variation in CRISPLD1 contributes to NSCLP and that both CRISPLD genes interact with each other and genes in the folic acid pathway. SNPs in CRISPLD1 were genotyped in our nonHispanic white and Hispanic multiplex and simplex NSCLP families. There was little evidence for a role of variation for CRISPLD1 alone in NSCLP. However, interactions were detected between CRISPLD1/CRISPLD2 SNPs and variation in folate pathway genes. Altered transmission of one CRISPLD1 SNP was detected in the nonHispanic white simplex families. Importantly, interactions were detected between SNPs in CRISPLD1 and CRISPLD2 (15 interactions, $0.0031 \leq p < 0.05$). These novel findings suggest that CRISPLD1 plays a role in NSCLP through the interaction with CRISPLD2 and folate pathway genes.

INTRODUCTION

Nonsyndromic cleft lip with or without cleft palate (NSCLP) is a common birth anomaly with a prevalence of 1/700 live births (Gorlin, 2001; Hashmi et al., 2005). This translates

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into approximately 4000 newborns with NSCLP each year in the United States. Treatment of this disorder requires a multidisciplinary approach involving genetics, surgical, dental and speech specialties. The cost of treatment is estimated at more than $100,000 per patient which presents a significant health care burden for these families (CDC, 1992).

The etiology of NSCLP is complex with both environmental and genetic factors causally implicated (Lidral and Moreno, 2005). For example, prenatal exposure to some antiepileptic medications increases the risk of NSCLP (Artama et al., 2005; Azarbayjani and Danielsson, 2001). Studies evaluating the teratogenic effects of prenatal exposure to either tobacco smoke or folate deficiency have yielded inconsistent outcomes (Blanton et al., 2000; Hecht et al., 2002; Shaw et al., 2002; Shaw et al., 1998; Shaw et al., 1999; Shaw et al., 1996). While there is increasing evidence supporting a genetic basis for NSCLP, only a small number of genes have consistently been implicated. For example, coding mutations in interferon regulatory factor 6 (IRF6) cause Van der Woude and Popliteal Pterygium syndromes, both of which have orofacial clefts as part of the phenotype. Recently, disruption of an AP2α binding site 14kb 5' of the IRF6 gene was shown to interfere with IRF6 regulation and this variant was associated with NSCLP (Rahimov et al., 2008). Based on studies in numerous populations, it is estimated that variation in IRF6 accounts for approximately 12% of NSCLP (Blanton et al., 2005; Scapoli et al., 2005; Zucchero et al., 2003; Zucchero et al., 2004). Other genes have also been implicated including BCL3, MSX1, MYH9, p63, RARA, TGFα, TGFβ and the WNT family (Amos et al., 1996a; Amos et al., 1996b; Chiquet et al., 2007; Lidral and Moreno, 2005; Marazita et al., 2004; Martinelli et al., 2007; Shaw et al., 1993; Stein et al., 1995; Suzuki et al., 2004). However, these associations have been inconsistent, account for an even smaller amount of the underlying genetic variation individually, and altogether explain very little of the genetic susceptibility in NSCLP.

Recently, we found an association between the cysteine-rich secretory protein LCCL domain containing 2 (CRISPLD2) gene and NSCLP and showed that CRISPLD2 was expressed in the developing murine craniofacial region (Chiquet et al., 2007). A second CRISPLD gene, CRISPLD1, shares 70% homology at the nucleotide level and 58% at the peptide level with CRISPLD2 (Kent et al., 2002). However, the function of CRISPLD1 is unknown. Both CRISPLD1 and CRISPLD2 contain more cysteine residues (25 and 26, respectively) when compared to the average cysteine composition of comparable sized proteins (5% vs. <2%) (White, 1992). Cysteine, a nonessential amino acid, is synthesized in the folate pathway (Aguilar et al., 2004). Genes in this pathway have been of interest in the study of birth defects because periconceptional folic acid usage decreases the birth prevalence of neural tube defects up to 70% (1991; Berry et al., 1999; Bower et al., 2009; Czeizel and Dudas, 1992; Lumley et al., 2001; MRC, 1991; Sayed et al., 2008). Likewise, the recurrence rate of orofacial clefting has been reduced in mothers taking higher doses of periconceptional folate but the reduction of NSCLP on a population level has been modest (Badovinac et al., 2007; Bille et al., 2007; Canfield et al., 2005; Czeizel et al., 1996; Hashmi et al., 2005; Shaw et al., 1995; Tolarova and Harris, 1995; van Rooij et al., 2003). The 5,10-methylenetetrahydrofolate reductase (MTHFR) gene regulates homocysteine levels and two nonsynonymous coding polymorphisms *C677T* (alanine to valine) and *A1298C* (glutamate

to alanine) affect the enzymatic activity (Jugessur et al., 2003; Weisberg et al., 1998). Studies assessing these MTHFR variants find minimal if any association with NSCLP (Blanton et al., 2000; Blanton, 2010a; Frosst et al., 1995; Goyette et al., 1994; Hecht et al., 2002; Jugessur et al., 2003; Shaw et al., 1998; Shaw et al., 1999; van der Put et al., 1998; Weisberg et al., 1998). However, a new study finds evidence suggesting that the folate pathway genes have a role in NSCLP (Blanton, 2010b).

Perturbation of the folate pathway could affect the production of cysteines with a resulting downstream effect on the synthesis and/or function of the CRISPLD genes, both of which require a large number of cysteine residues. Here, we asked if CRISPLD1 was associated with NSCLP and then tested whether CRISPLD1, CRISPLD2 and folate pathway genes interact to create a susceptibility to NSCLP.

METHODS

Study population and sample collection

The study population consisted of 107 and 291 nonHispanic white and 41 and 127 Hispanic families with positive (multiplex) and negative (simplex) histories of NSCLP, respectively. Study participants were ascertained at either the University of Texas Craniofacial Clinic, Houston, Texas Children's Hospital, Houston or Children's Hospital, Boston as previously described (Chiquet et al., 2007). The institutional review boards at all institutions approved this study. Blood and/or saliva samples were collected from each participant. DNA was extracted from blood using Roche DNA Isolation Kit for Mammalian Blood (Roche, Switzerland) and from saliva using Oragene Purifier (DNA Genotek, Inc., Ontario, Canada) following manufacturer's protocol.

Genotyping

Nine single nucleotide polymorphisms (SNPs) in CRISPLD1 (Table 1) were selected using previously described criteria and genotyped using TaqMan® SNP Genotyping assays [Applied Biosystems (AB), Foster City, CA] (Chiquet et al., 2007). Allele calls were generated using the AB 7900HT Sequence Detection System.

Ninety-seven SNPs spanning 14 folate pathway genes were interrogated and included: Betaine-homocysteine methyltransferase (BHMT, 6 SNPs), BHMT2 (6 SNPs), Cystathionine-beta-synthase (CBS, 12 SNPs), Folate receptor 1 (FOLR1, 4 SNPs), FOLR2 (4 SNPs), Methylenetetrahydrofolate dehydrogenase 1 (MTHFD1, 12 SNPs), MTHFD2 (4 SNPs), 5,10-methylenetetrahydrofolate reductase (MTHFR, 8 SNPs), 5,10-methylenetetrahydrofolate synthase (MTHFS, 4 SNPs), Methylenetetrahydrofolate dehydrogenase (MTR, 9 SNPs), 5-methyletetrahydrofolate-homocysteine methyltransferase reductase (MTRR, 3 SNP), ATG9 autophagy related 9 homolog (NOS3, 6 SNPs), Solute carrier family 19, member 1 (SLC19A1, 10 SNPs) and Tymidylate synthetase (TYMS, 9 SNPs) (Blanton, 2010b). All SNPs were genotyped using the ABI SNPlex™ Genotyping System following manufacturer's protocol and are described in detail in Blanton et al. 2010. Allele calls were generated using the AB 3730 and AB GeneMapper® Software v4.0.

Data from both ABI TaqMan® and ABI SNPlex™ genotyping assays were entered into Progeny Lab (South Bend, IN) and assessed for Mendelian errors using PedCheck (O'Connell and Weeks, 1998).

Analysis

CRISPLD1 allele frequencies were generated and tested for Hardy-Weinberg Equilibrium (HWE) using SAS (v9.1). GOLD (graphical overview of linkage disequilibrium) analysis was used to assess linkage disequilibrium (D' and r^2 values) (Abecasis and Cookson, 2000). Parametric and nonparametric analysis was performed using MERLIN (multipoint engine for rapid likelihood inferences) with linkage parameters as previously described (Abecasis et al., 2002; Blanton et al., 2004). Pedigree disequilibrium test (PDT), genotype-PDT (G-PDT), association in the presence of linkage (APL) and family based association test (FBAT-e) were used to detect association (Chung et al., 2006; Martin et al., 2003; Martin et al., 2000). The generalized estimating equations (GEE) algorithm was used to assess gene interactions (Hancock et al., 2007). Complete results for the CRISPLD2 association study is presented in Chiquet et al. (Chiquet et al., 2007). Analysis of the folate pathway genes is found in Blanton et al. (Blanton, 2010b).

RESULTS

Nine CRISPLD1 SNPs (5 intragenic, 4 intergenic; Fig. 1, Table 1) were genotyped in our nonHispanic white and Hispanic families. All SNPs had >95% call rate and were in HWE. The data was stratified by ethnicity because the allele frequencies for five of the nine SNPs differed between the nonHispanic white and Hispanic groups ($p \leq 0.006$). The data was also stratified by the presence or absence of family history (FH) (Lewis et al., 1987). Significant linkage disequilibrium was found in both datasets ($r^2 > 0.95$; Supplemental Table 1). A maximum LOD score of 1.35 was found to CRISPLD1 SNPs in nonHispanic whites (data not shown). There was no evidence of linkage in the Hispanic dataset.

Association analysis of the single SNPs identified altered transmission for only rs1455809 in the nonHispanic white simplex families ($p=0.05$) (Supplemental Table 2). No single SNP associations were detected in the Hispanic group. None of the CRISPLD1 2-SNP haplotypes demonstrated altered transmission in either ethnicity (data not shown).

Three gene-gene interactions were detected between CRISPLD1 and CRISPLD2 SNPs ($0.003 \leq p < 0.01$) (Table 2). The same CRISPLD1 SNPs in nonHispanic white and Hispanics interacted with different CRISPLD2 SNPs in each ethnic group (Table 2). The most significant interactions involved rs13248650–rs12051468 ($p=0.004$) and rs13248650-rs8051428 ($p=0.003$) in the nonHispanic White and Hispanic groups, respectively.

GEE analysis identified numerous interactions between SNPs in CRISPLD1 and CRISPLD2 and the folate pathway genes. In the nonHispanic white dataset, SNPs in five of the folate pathway genes interacted with SNPs in CRISPLD1. The most significant interaction was between rs7166109 in MTHFS and rs10957748 ($p=0.0005$) (Table 3). rs7166109/MTHFS interacted with four other CRISPLD1 SNPs ($p \leq 0.002$). Three different CBS SNPs interacted with four different CRISPLD1 SNPs; rs1455796/CRISPLD1 interacted with two different

CBS SNPs (Table 3). In the Hispanic dataset, the most significant CRISPLD1 interactions involved SNPs in MTR ($0.001 \leq p \leq 0.005$) (Table 3). Additionally, two different SNPs in TYMS interacted with different CRISPLD1 SNPS in each ethnic group.

Numerous interactions between CRISPLD2 and folate pathway genes were detected in both datasets (Table 4). In the nonHispanic whites, there were seventeen SNPs in eleven folate pathway genes interacting with CRISPLD2 SNPs. The most significant interaction was with BHMT2 ($0.0005 \leq p \leq 0.003$). Two MTR SNPs, rs1546124 and rs1874015, also interacted with CRISPLD2 ($0.0009 \leq p \leq 0.007$). In the Hispanics, there were ten SNPs in six folate pathway genes interacting with CRISPLD2. The most significant interactions were found for three SNPs in MTHFR ($0.003 \leq p \leq 0.007$). Interestingly, in both groups, rs2236222/MTHFD1 interacted with rs12051468/CRISPLD2 ($0.003 \leq p \leq 0.008$). Furthermore, for both ethnic groups, SNPs in TYMS, MTHFR and FOLR1 interacted with CRISPLD2 SNPs.

DISCUSSION

In previous studies, we reported the association of variation in CRISPLD2 with NSCLP and showed that CRISPLD2 was expressed in the craniofacies of developing mouse embryos at E13.5 (Chiquet et al., 2007). This association has been confirmed in an independent dataset (Letra et al., 2010 in press). In our on-going studies to define the genetic loci contributing to NSCLP, 11 chromosomal regions with LOD scores ≥ 1.5 were identified in a genome scan performed on nine multiplex nonHispanic white NSCLP families (Chiquet et al., 2009). A LOD score of 1.23 was found for the 8q13.2-21.13 chromosomal region which contains the CRISPLD1 gene; CRISPLD1 has significant homology to CRISPLD2 and both genes are composed of more cysteines than comparable-sized proteins (5% protein composition vs. 2%) (White, 1992). The cysteines provide secondary structure to the protein backbone and help protect cells against the harmful effects of oxidation (Benard and Balasubramanian, 1993; Saitão, 1989). These interesting results led to the interrogation of CRISPLD1 as a candidate NSCLP gene. In complementary studies, we show that variation in different folate pathway genes individually and through interactions contribute to NSCLP (Blanton, 2010b). Interestingly, cysteines, which could potentially be utilized by the CRISPLD genes, are produced in the homocysteine biosynthesis pathway, which is part of the folate pathway (Boyles et al., 2008). Based on these observations, we asked whether the CRISPLD genes interacted with genes in the folate pathway.

We show that only one CRISPLD1 SNP, rs1455809, had marginally altered transmission ($p=0.05$) suggesting that variation in CRISPLD1 alone does not play a significant etiologic role in NSCLP. Moreover, there were only three CRISPLD1-CRISPLD2 interactions (two in nonHispanic whites and one in Hispanics) suggesting that these genes do not functionally interact to create a susceptibility to NSCLP. Indeed, their expression patterns in mice are different. At E14.5, CRISPLD1 is expressed in the mouse brain, spinal cord, nose, alimentary system, respiratory system, skeleton and limbs, while CRISPLD2 is expressed in the oral region, visceral organs, alimentary system and salivary gland (www.euroexpress.org/ee/) (Chiquet et al., 2007). In zebrafish, CRISPLD2 is expressed in the craniofacial region and tail at all stages of development while CRISPLD1 localizes to

the splanchocranium, pectoral fin, presumptive vertebrate and epiphysis (Chiquet and Hecht, unpublished results) (Thisse, 2004).

Folic acid is important in embryogenesis and this is underscored by the 70% reduction of neural tube defects since folic acid supplementation of grain products in 1998 (Canfield et al., 2005; Honein et al., 2001; MMWR, 2004; Sayed et al., 2008). While a similar reduction has not been observed for NSCLP on a population-basis, decreased recurrence of NSCLP has been observed when high-risk mothers take high dose folic acid pre- and post conception (Berry et al., 1999; Czeizel et al., 1996; Hashmi et al., 2005; Shaw et al., 1995; Tolorova and Harris, 1995). This suggests that perturbation of folic acid levels or genes in the folate pathway could contribute to NSCLP. To evaluate this question, we first assessed whether folate pathway genes were associated with NSCLP (Blanton, 2010b). Evidence for an association was found for SNPs in NOS3, TYMS, MTR, BHMT2, MTHFS and SLC19A1; many of the associated variants occurred in potential promoter or regulatory regions. Complete description of the results is found in Blanton et al. 2010. The endogenous folate pathway is responsible for synthesizing cysteine and cysteine is required for the CRISPLD1/2 protein structures. This led us to postulate that CRISPLD genes interact with genes in the folate pathway. Indeed, we found a number of important interactions. In the nonHispanic whites, rs502396 in TYMS interacted with CRISPLD1 and this SNP had altered transmission in the single SNP folate pathway gene analysis (Blanton, 2010a; b). Similarly, for CRISPLD2, two different SNPs in TYMS, rs11540152 and rs2853532, interacted although these SNPs did not have altered transmission in the folate study. Additionally, rs2373929 in NOS3 interacted with CRISPLD2 and this SNP also exhibited altered transmission in the single SNP folate pathway gene analysis. In the Hispanics, three MTR SNPs interacted with CRISPLD1 but they were different from the MTR SNPs identified in the single SNP folate analysis. For CRISPLD2, rs3788203 in SLC19A1, which was associated in the single folate SNP analysis, also showed interaction. These results are particularly interesting because, as shown in Fig 2, MTR is necessary to metabolize homocysteine to methionine, a process that generates cysteines as a byproduct. NOS3 directly regulates MTR and SLC19A1 affects 5mTHF, an intermediary that is also metabolized by MTR. Additionally, interactions with both CRISPLD genes were found for CBS, BHMT and BHMT2 in the nonHispanic white dataset. These genes also participate in the methionine cycle. Other interactions were found for both CRISPLD genes and TYMS and MTHFS, both of which participate in the DNA synthesis necessary for embryonic development.

Altogether, these results suggest that CRISPLD genes play a role in NSCLP but not through a simple mechanism. We have shown that variation in CRISPLD2 is associated with NSCLP but CRISPLD1 alone is not. A more likely mechanism that is suggested by these results is that perturbation in multiple genes in a pathway affects protein function, which can have a profound effect on embryogenesis. Indeed, we have found the same etiologic model for nonsyndromic clubfoot, which is another common complex birth defect (Ester et al., 2009). These results would also fit what is known about recurrence in NSCLP wherein high dose folic acid reduces the recurrence in high-risk families. These families may have more liability genes that could potentially respond to drug/vitamin therapy. In contrast, on a population basis, genetic heterogeneity may play a role and folate deficiency may only be a

small piece of the puzzle. Thus additional studies are necessary to define all the genetic contributions. These results are intriguing and require both validation and functional studies from which we may be able to build high risk haplotypes in order to provide better genetic counseling and perhaps population screening.

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Table 1

CRISPLD1 SNP alleles and frequencies by ethnicity

dbSNP	Basepair	Alleles[a]	Non-Hispanic White MAF[b]	Hispanic Corresponding Frequency[c]	p-value[d]
rs2925155	76048852	A/G	0.263	0.317	0.033
rs960856	76057462	T/A	0.329	0.248	0.002*
rs7841231	76065317	A/G	0.321	0.232	0.0005*
rs17295835	76069196	T/C	0.299	0.354	0.035
rs1455809	76075622	T/C	0.312	0.234	0.002*
rs1455796	76086362	C/G	0.405	0.411	0.804
rs10957748	76101956	C/T	0.292	0.210	0.001*
rs13248650	76109250	T/G	0.300	0.362	0.019
rs11988595	76115241	C/T	0.310	0.233	0.003*

[a] Most common allele listed first

[b] Minor allele frequency

[c] Frequency in Hispanic of non-Hispanic white minor allele

[d] p-values less than Bonferoni correction of 0.0056 denoted with *

Table 2

CRISPLD1-CRISPLD2 Gene Interactions

Ethnicity	CRISPLD1/SNP	CRISPLD2/SNP	p-value*
nonHispanic white	rs13248650	rs12051468	**0.004**
	rs2925155	rs12051468	**0.010**
	rs960856	rs903194	0.012
	rs7841231	rs903194	0.015
	rs13248650	rs8051428	0.022
	rs17295835	rs12051468	0.023
	rs10957748	rs12051468	0.039
	rs10957748	rs903194	0.043
Hispanic	rs13248650	rs8051428	**0.003**
	rs17295835	rs8051428	0.018
	rs7841231	rs2646112	0.02
	rs17295835	rs2646112	0.022
	rs13248650	rs2646112	0.024
	rs960856	rs721005	0.024
	rs7841231	rs721005	0.03

* $p<0.05$, $p<0.01$ bolded

Table 3

Folate Pathway and CRISPLD1 gene interactions

Ethnicity	Gene/SNP	CRISPLD1 SNP	p-value*
nonHispanic white	CBS/rs234783	rs1455796	0.004
		rs13248650	0.005
		rs17295835	0.006
	CBS/rs2851391	rs2925155	0.007
	CBS/rs12329790	rs1455796	0.008
	MTHFS/rs7166109	rs10957748	0.0005
		rs960856	0.001
		rs7841231	0.001
		rs11988595	0.001
		rs1455809	0.002
	TYMS/rs502396	rs10957748	0.01
Hispanic	MTR/rs1266164	rs1455796	0.001
	MTR/rs12354209	rs1455796	0.004
	MTR/rs1806505	rs10957748	0.005
	TYMS/rs11540152	rs1455796	0.004

* p-value≤0.01

Table 4

Folate Pathway and CRISPLD2 Gene Interactions

Ethnicity	Gene/SNP	CRISPLD2 SNP	p-value*
nonHispanic white	BHMT/rs645112	rs1874014	0.009
	BHMT/rs3733890	rs16974880	0.01
	BHMT2/rs2253262	rs1874014	0.0005
	BHMT2/rs682985	rs1874014	0.001
	BHMT2/rs1422086	rs1874014	0.003
	FOLR1/rs3016432	rs2646112	0.007
	FOLR2/rs2276048	rs2646112	0.005
	MTHFD1/rs2236222	rs12051468	0.008
	MTHFD2/rs7587117	rs2641670	0.003
	MTHFR/rs1476413	rs8051428	0.007
	MTHFS/rs2586179	rs1874015	0.003
		rs767050	0.009
	MTR/rs12354209	rs1546124	0.0009
		rs1874015	0.007
	MTR/rs1266164	rs1546124	0.004
	NOS3/rs1800779	rs16974880	0.005
	NOS3/rs2373929	rs16974880	0.005
	TYMS/rs11540152	rs2646129	0.008
	TYMS/rs2853532	rs12051468	0.010
Hispanic	CBS/rs2851391	rs2646112	0.009
	FOLR1/rs2071010	rs774206	0.006
		rs721005	0.009
	MTHFD1/rs2236222	rs12051468	0.003
	MTHFD1/rs11849530	rs2641674	0.006
	MTHFR/rs1801131	rs2646112	0.003
	MTHFR/rs1476413	rs2646112	0.003
	MTHFR/rs535107	rs8061351	0.007
	SLC19A1/rs3788205	rs4783099	0.01
	TYMS/rs495139	rs8061351	0.002
	TYMS/rs1001761	rs8061351	0.003

* p-value≤0.01