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ACSS2 gene variant associated with cleft lip and palate in two independent Hispanic populations

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Abstract

Objectives—A candidate variant (p.Val496Ala) of the *ACSS2* gene (T>C missense, rs59088485 variant at chr20: bp37 33509608) was previously found to consistently segregate with non-syndromic cleft lip and/or palate (NSCLP) in three Honduran families. Objectives of this study were (1) investigate the frequency of this *ACSS2* variant in Honduran unrelated NSCLP patients and unrelated unaffected controls and (2) investigate the frequency of this variant in Colombian unrelated affected NSCLP patients and unrelated unaffected controls.

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Methods—Sanger sequencing of 99 unrelated Honduran NSCLP patients and 215 unrelated unaffected controls for the p.Val496Ala *ACSS2* variant was used to determine the carrier frequency in NSCLP patients and controls.

Sanger sequencing of 230 unrelated Colombian NSCLP patients and 146 unrelated unaffected controls for the p.Val496Ala *ACSS2* variant was used to determine the carrier frequency in NSCLP patients and controls.

Results—In the Honduran population, the odds ratio of having NSCLP among carriers of the p.Val496Ala *ACSS2* variant was 4.0 (p = 0.03) with a carrier frequency of 7/99 (7.1%) in unrelated affected and 4/215 (1.9%) in unrelated unaffected individuals.

In the Colombian population, the odds ratio of having NSCLP among carriers of the p.Val496Ala ACSS2 variant was 2.6 (p value 0.04) with a carrier frequency 23/230 (10.0%) in unrelated affected and 6/146 (4.1%) in unrelated unaffected individuals.

Conclusions—These findings support the role of *ACSS2* in NSCLP in two independent Hispanic populations from Honduras and Colombia.

Keywords

ACSS2; non-syndromic cleft lip and/or palate; Honduras; Colombia; Amerindian; cleft; lip; palate

Introduction

Cleft lip and/or palate (CLP) is relatively common, occurring in about 1/700 live births worldwide[1]. Of note, there is variability in prevalence amongst different geographical, ethnic, and racial groups, with the highest prevalence occurring in Asian and Amerindian populations, at a frequency of 1/500[1]. CLP poses a considerable burden on families and society at large, as CLP patients require multidisciplinary care until adulthood to address defects in hearing, speech, dentition, nutrition, appearance, and mental health[2]; ultimately, they suffer higher morbidity and mortality throughout their lives[3].

CLP is classified as either non-syndromic cleft lip and/or palate (NSCLP), which accounts for about 70% of cases[4] or syndromic cleft lip and/or palate, which occurs with established syndromes such as the Van der Woude, Pierre Robin, and Velocardiofacial syndromes[5]. NSCLP is of particular interest as it represents the majority of CLP cases and is thought to occur secondary to a complex host of genetic and environmental factors[6], many of which are yet to be elucidated.

Genome wide association studies (GWAS) have successfully identified numerous common variants associated with NSCLP[7] while whole exome sequencing (WES) has been used to identify very rare genetic variants associated with oral clefts[8]. Prior work utilized WES to identify a candidate variant (p.Val496Ala) in the *ACSS2* gene that consistently segregates with NSCLP in three Honduran multiplex families[9]. This gene is of particular interest in oral clefts, as it has been found to be involved in mouse cephalic development[10].

The current study objective is to investigate the frequency of the *ACSS2* p.Val496Ala variant in two independent Hispanic populations (Hondurans and Colombians) in unrelated NSCLP

patients and unrelated unaffected controls via separate case control studies in each population.

Materials and Methods

Human Subjects

Honduran subjects were identified at Hospital Escuela in Tegucigalpa, Honduras. The Honduran subjects included 99 unrelated NSCLP patients, and 215 unrelated unaffected controls. Subjects were excluded if they had syndromic characteristics as determined by family history and physical examination (lip pits in Van der Woude; micrognathia, glossoptosis, or retrognathia in Pierre Robin; congenital heart disease or abnormal facial characteristics in Velocardiofacial). Honduran controls were sex-matched pediatric patients undergoing minor surgical procedures and were excluded if they had a personal or family history of clefting, or if they had any genetic or congenital diseases. Physical exam was performed to assess for clefting in cases and controls, and included visual inspection of the lips and visual inspection of the entire mouth by using a bright light and a tongue depressor to press the child's tongue to the floor of the mouth. Subclinical features of NSCLP assessed on physical exam included a ridge of tissue on the lips and anomalies in dentition. In addition, a gloved finger was placed in the mouth to palpate defects in the palate. Written informed consent and assent were obtained from Honduran subjects. Venous blood was drawn from subjects and pedigrees were constructed. This study was approved by the institutional review board of the Columbia University Medical Center.

Colombian subjects were recruited in the past six consecutive years during the annual Smile Train Mission at Hospital Universitario Hernando Moncaleano Perdomo in Neiva, Colombia. The Colombian subjects included 230 unrelated affected NSCLP patients and 146 unrelated unaffected controls. Each family was clinically characterized and verified in the Smile Train Express database. Collection of biospecimens, medical records, photographs, family history, and pedigrees were taken from each individual. Colombian controls were sex-matched and excluded if they had a personal or family history of clefting, or any congenital or genetic diseases. Written informed consent and assent were obtained from Colombian subjects under the protocol from Universidad Surcolombiana, in regulation and approved by Columbia University's IRB.

SNP Genotyping

Genomic DNA was isolated from whole blood samples with Qiagen Flexigene kits (Qiagen, Valencia, CA). One candidate variant (p.Val496Ala; T>C missense, rs59088485, at chr20: bp37 33509608) in the *ACSS2* gene was chosen for these case control studies since it had been found to consistently segregate with NSCLP in three Honduran multiplex families in a previous study[9]. This candidate variant is predicted to have damaging characteristics, specifically a SIFT score of 0.01, PolyPhen2 of 0.999, and a Combined Annotation–Dependent Depletion (CADD) score of 25.9. SIFT and PolyPhen2 both use different algorithms to assess the damaging nature of an amino acid substitution on protein structure and function; SIFT spans 0 to 1, with 0 being the most deleterious[11] while PolyPhen2 also runs from 0 to 1, but with a score of 1 being the most damaging[12]. The CADD score

aggregates information from a number of different predictors of the damaging nature of mutations and a score of >20 indicates that a variant is in the top 1% of most deleterious variants in the human genome[13]. To briefly summarize, the preceding describes that this candidate variant is a nucleotide change (T > C) with a resultant missense change (amino acid substitution of Valine > Alanine) that has been predicted to have a highly damaging effect on protein structure and function based on multiple commonly used algorithms. It was also found to be rare in the publicly available population databases, with a frequency in 1000 genomes of 0.01 and exome aggregation consortium of 0.00185[9].

Primer3[14] was used to amplify 400–600 base pair regions. PCR products were sequenced by Macrogen (www.macrogenusa.com) and chromatogram results were viewed with FinchTV (Version 1.4 http://www.geospiza.com/Products/finchtv.shtml).

Hondurans—Sanger sequencing was performed on the genomic DNA isolated from 99 unrelated affected Honduran NSCLP patients and 215 unrelated unaffected Honduran controls.

Colombians—Sanger sequencing was performed on 230 unrelated affected Colombian NSCLP patients and 146 unrelated unaffected Colombian controls.

Statistical Analysis

We determined odds ratios for the p.Val496Ala *ACSS2* variant in unrelated affected NSCLP patients vs. unrelated unaffected controls for Honduran and Colombian populations (in separate analyses for each population).

Results

The results of this study (carrier frequency, odds ratio, confidence interval, and p-value) are shown in Table 1. The carrier frequency of p.Val496Ala in the Honduran population was 7/99 (7.1%) amongst unrelated affected NSCLP patients and 4/215 (1.9%) amongst unrelated unaffected controls, with an odds ratio of 4.0 (p value 0.03) and a confidence interval of 1.1 to 14.0. In the Colombian population, the p.Val496Ala carrier frequency was 23/230 (10.0%) amongst unrelated affected NSCLP patients and 6/146 (4.1%) amongst unrelated unaffected controls, with an odds ratio of 2.6 (p value 0.04) and a confidence interval of 1.0 to 6.5.

Discussion

We previously identified p.Val496Ala in *ACSS2* as a variant contributing to NSCLP based upon segregation data in three multiplex NSCLP Honduran families. This current study was designed to study the association of the p.Val496Ala variant with NSCLP in two independent Hispanic populations from Honduras and Colombia to provide independent evaluation of the variant. This study demonstrates that the p.Val496Ala variant in *ACSS2* is significantly more prevalent in NSCLP patients than in controls in two independent Hispanic populations as supported by an odds ratio of 4.0 (p value 0.03) and 2.6 (p value 0.04) in Hondurans and Colombians, respectively, of variant carriers having NSCLP.

ACSS2 is one of three genes (*ACSS1*, *ACSS2*, and *ACSS3*)[15] that codes acetyl-CoA synthetase short chain, a ligase enzyme that catalyzes the ATP-dependent ligation of acetate and coenzyme A (CoA) to form acetyl-CoA, which can be used for lipid synthesis or energy production[16]. Typically, ACSS1 is located in the mitochondria, ACSS2 in the cytosol, and ACSS3 in the mitochondria[17]; however, ACSS2 has been found in the mitochondrial matrix as well[18]. During nutrient poor conditions, acetate (rather than glucose) serves as a main source of acetyl-CoA[19]; consequently, mutations of *ACSS2* may affect cell growth and survival in conditions with nutritional (metabolic) stressors.

Most studies to date focusing on *ACSS2* and nutritional (metabolic) stressors have been done in the context of tumorigenesis. It has been demonstrated that ^{13}C labeled acetate is elevated in nutrient poor conditions[20] and in patient derived xenograft tumors[21], suggesting that acetate becomes a more important nutrient source during nutritional (metabolic) stress. In addition, *ACSS2* has been found to be upregulated to maintain acetate utilization during metabolic stress, such as during cancer cell growth[21] and lipid-depleted conditions[20], indicating that *ACSS2* plays an increasingly important role during nutritional (metabolic) stress.

Other studies have investigated the underlying mechanism of ACSS2's role in nutrient sensing and have distinguished ACSS2 amongst the other ACSS isoforms. With regard to the underlying mechanism, a study noted that hypoxia inducible factor 2 (HIF-2), a transcription factor in a signaling pathway with ACSS2, is activated during glucose deprivation, and demonstrated that this signaling pathway (ACSS2/creb binding protein (CBP)/sirtuin 1 (SIRT1)/HIF-2) plays a role in nutrient sensing during cancer growth in mammals[22]. Lastly, a recent study examined the role of ACSS2 in acetate utilization in the context of the other isoforms of the enzyme. Specifically, amongst the three ACSS enzymes (ACSS1, ACSS2, and ACSS3), ACSS2 was found to have the greatest effect on acetate utilization in liver tumor cells, and mice with ACSS2 knockdowns had significantly lower tumor burdens than control mice[23]. Taken together, these studies all point to a role of *ACSS2* in promoting cell growth and survival in nutrient-stressed conditions.

In addition, the *ACSS2* gene is involved in development, including cephalic development; this role in development coupled with *ACSS2*'s role in cell growth during nutrient-stressed conditions may reveal a potential link between *ACSS2* and clefting. Acetyl-CoA synthetase is involved in early implantation[24] and the early spontaneous differentiation of embryonic stem cells[25]. The concentration of acetyl-CoA synthetase increases throughout fetal development[26] and has intensified expression in the cephalic region, especially the forebrain region, during the second week of mouse development[10]. Taken together, *ACSS2* plays a role in cephalic development and in cell growth and survival during nutrient-stressed conditions. Given the potential presence of nutritional (metabolic) stressors during development, mutations in *ACSS2* may predispose cells to difficulty responding to such stressors and may pose an impediment to normal craniofacial development.

It is important to note that this candidate variant was found in unaffected controls, and there are two likely explanations for this. First, a previous study[9] described this candidate variant as having incomplete penetrance; thus, controls carrying this candidate variant may have had

subclinical signs of cleft lip/palate (i.e. defects of the orbicularis oris muscle). Alternatively, the controls carrying this candidate variant may have been completely unaffected. If that is the case, it may be that there is an interplay of genetic modifiers, epigenetic regulation and/or other genes in addition to this candidate variant on *ACSS2* that is responsible for the association between *ACSS2* and cleft lip/palate.

Another important point to consider is the possible Mayan ancestry of the Honduran and Colombian patients. The Honduran population is considered to be homogenous, with the majority being mestizo (mixed European and Amerindian descent). Similarly, the majority of Colombians are either mestizo or of European descent. It is worth noting that Mayans have been found to have higher rates of CLP[27,28] and a population-based genetic study focusing on the Caribbean has demonstrated that native Mayan, Honduran, and admixed Colombians share genetic components[29]. Though we did not specifically study Mayan ancestry in this present study, further studies may investigate the role of Mayan genetic influence on other Caribbean and South American populations, specifically as it pertains to cleft lip/palate.

The strength of this study is the inclusion of genetic data from two independent Hispanic populations. The limitations include the modest sample size and the possible inadvertent omission of subclinical features of NSCLP, though the subjects in both populations did undergo a thorough physical examination to assess for subclinical features of NSCLP. Future directions include the assessment of the association of this candidate variant of *ACSS2* in additional Hispanic populations, and additional populations of other ethnicities. In addition, it would be worthwhile to investigate the genetic modifiers and epigenetic regulation of *ACSS2*, as well as the interplay of other genes with *ACSS2*.

Conclusion

This study supports previous findings regarding the role of the *ACSS2* gene in NSCLP and is the first to demonstrate association of this gene in NSCLP in two independent Hispanic populations. Additional studies should further characterize the role of this gene in these two populations and assess the association between variants in this gene and NSCLP in other Hispanic populations.

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Table 1

Honduran and Colombian Case-Control study of the p.Val496Ala *ACSS2* variant: carrier frequencies, odds ratios, and p-values.

	Hondurans		**Colombians**	
	Cases	**Controls**	**Cases**	**Controls**
Carrier frequency	7/99 (7.1%)	4/215 (1.9%)	23/230 (10.0%)	6/146 (4.1%)
Odds ratio (95% Confidence Interval)	4.0 (1.1 to 14.0)		2.6 (1.0 to 6.5)	
P-value	0.03		0.04	