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CRC Research Protocol
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1) Analysis of Folic Acid Metabolism Loci Associating with Non-syndromic Cleft Lip with or without Cleft Palate in a Honduran Population
2) Exome Sequencing to Detect Rare Mutations Associated with Non-syndromic Cleft Lip with or without Cleft Palate in a Honduran Population

A. Study Purpose and Rationale

Cleft of the lip with or without cleft palate (CL/P) and isolated cleft palate (CP) represent the most common congenital craniofacial defects in humans with an average worldwide incidence ranging from 1 to 7 in 1000 live births and wide variability across geographic origin and ethnic groups[1]. Asian and Native American populations have the highest reported prevalence rates, often reported as high as 1 in 500[2]. Orofacial clefting (OFC) causes considerable morbidity, including speech difficulties, malnutrition, hearing impairment, infection, and psychiatric disease. While these defects can often be corrected by surgery, dentistry, speech therapy, and psychosocial intervention using a multidisciplinary approach to treatment, clefting nonetheless imposes a substantial financial risk for families and society[3].

CL/P may occur in an isolated, non-syndromic form or as a part of a complex malformation syndrome, with non-syndromic cleft lip with or without cleft palate (NSCLP) constituting the majority (~70%) of cases[4]. With the advent of the genomics era, many advances have been made towards identification of causative genetic mutations underlying syndromic forms of CLP[ii]. However, whereas twin studies and familial clustering studies have provided evidence for a genetic component to NSCLP, it shows a departure from Mendelian inheritance patterns in most pedigrees. Moreover, NSCLP is also known to be influenced by environmental risk factors as well[5]. Thus, current evidence suggests non-syndromic clefting has a multifactorial etiology, with both genetic and environmental contributions[1].

Steady progress towards the identification of genes contributing to this disorder has been made over the past decade, with a number of susceptibility genes uncovered. Recently, a number of Genome-Wide Association (GWA) studies have identified genetic susceptibility factors for NSCLP[6,7,8,9]. These studies suggested potential roles for multiple single nucleotide polymorphisms (SNPs), including those in and around *IRF6*, *MAFB*, *ABCA4*, 8q24, *FOXE1, NOG*, and *VAX*, in NSCLP in a multitude of populations. Three SNPs in the locus containing IRF6, 1q32.3 to 1q41, were previously demonstrated by our group to be significantly associated with NSCLP in the Honduran population[10]. Furthermore, a risk association in both case-control and family-based association studies was demonstrated between NSCLP and one SNP (rs2235371) that has been proposed to have biological significance to IRF6 expression and function, suggesting a role for this polymorphism in our Honduran population[11]. Our group has also previously identified SNPs in *ABCA4, FOXE1, MAFB,*

NOG, and 8q24 to be associated with NSCLP in a case-control model in our Honduran cohort (unpublished data).

Gene-environment interactions have also been suggested to contribute to clefting, with strong evidence for interaction between maternal smoking fetal variants of *GSTM1* and *GSTT1*[12,13]. Folate deficiency has also emerged as a possible environmental cause of OFC. It is well known that folate deficiency has been causally related to neural tube defects (NTDs). Although the underlying mechanism of this causal relationship remains to be defined, it is known that the neural tube and the craniofacial regions both arise from neural crest cells[14]. Thus, it has been hypothesized that folate deficiency may play a role in development of OFC as well. Some observational studies have reported a preventive effect of folic acid-containing supplements on OFC[15]. However, due to sample selection biases and differences in sample sizes/statistical power, populations, analytical models, and folic acid measures, the evidence of observational studies is mixed[16]. However, a metaanalysis of these studies estimated a reduction of approximately 28% and 20% in the risks of CL/P and CP respectively while using folic acid-containing supplements and/or multivitamins[17].

Numerous folate gene interaction studies have been conducted yielding often contradictory results. Methylene tetrahydrofolate reductase (*MTHFR*) is a gene that codes for a folate metabolizing enzyme and has been studied as a candidate gene. The T677C and A1298T variants of this gene have emerged as possible factors in the modification of effects of folic acid supplementation. However, numerous studies have not found an association with these variants[18,19,20,21]. The other main candidate genes involved in folate metabolism that have been studied include *MTHFD, MTR, MTRR, RFC1, GCP2, CBS, BHMT, BHMT2,* and *TS*[16]. In a recent comprehensive SNP analysis of 14 folate metabolism-related genes, evidence for a risk association between NSCLP and SNPs in *MTR, BHMT2, MTHFS,* and *SLC19A1* were detected in a Hispanic cohort[22], suggesting a role for these genes in OFCs in populations of similar ancestry.

The Honduran population is advantageous for this study due to its increased rate of clefting (given their Amerindian ancestry) as well as their relative genetic isolation. Our investigation will examine a cohort of multiplex (two or more members affected by clefting) families to evaluate the involvement of five candidate genes in cases of clefting in Honduras. Our study is restricted to multiplex families in order to heighten genetic influence. To date, these candidate genes have not been studied in the Honduran population.

Finally, recent advances in DNA analysis include the ability to select and sequence all the exons from a person's DNA. Since exons are responsible for 85% of disease-causing variants in DNA, but only compose 5% of each human's total DNA, this is a more efficient means to identify new mutations associated with NSCLP. We will use our pool of DNA from patients with NSCLP for exomic sequencing to determine new, rare variants in genes that may contribute to NSCLP. This is a novel method to

attempt to identify genes and variants that are responsible for cleft lip and palate in our Honduran population.

B. Study Design and Statistical Analysis

The proposed study is a joint case-control and family-based association study of genetic association with NSCLP. To date, we have recruited a total of 146 patients affected with NSCLP and 370 unaffected members from 105 families and 128 age and gender-matched controls. Families have been recruited for the study after presenting to a cleft clinic at Hospital Escuela, a public hospital in Tegucigalpa, Honduras. Each subject enrolled has been evaluated for type of cleft, laterality, palatal involvement, and additional craniofacial/organ system anomalies, thus excluding syndromic and isolated cleft palate presentations. Additionally, patients' mothers are interviewed to exclude use of medications associated with clefting phenotypes during pregnancy. For those affected by clefting, a thorough family pedigree is recorded and digitally calalogued into Cyrillic 3, a pedigree drawing program. The subjects are also logged into a Microsoft Access database to prevent redundant recruitment of subjects. Venipuncture is performed on as many family members as possible, making efforts to contact both parents in order to secure case-parent trio data for family-based association testing. If children are to undergo anesthesia, blood is drawn while the patient is unconscious to minimize psychological trauma. The blood samples and pedigree is then sent to Columbia University Medical Center for DNA extraction with the Qiagen Flexigene DNA kit and record keeping, respectively.

SNPs in *MTHFR, MTR, BHMT2, MTHFS,* and *SLC19A1* were selected based on heterozygosity ($p > 0.3$, where p is the high-risk allele frequency), functionality, intergenic and intragenic positions, and previously reported association with NSCLP in related populations. In total, 15 SNPs from these 5 genes will be analyzed. Even with a conservative estimate of $p = 0.10$, we can show that our sample size is sufficiently large to achieve 80% power for Armitage's test for trend for $\alpha = 0.05$ in the multiplicative, additive, and dominant models[23]. Given our relatively small SNP pool, a Bonferroni Correction is not needed in our analysis. In Family-Based Association testing, since the types of pedigrees and availability of blood samples varies greatly between pedigrees, power is difficult to calculate. Furthermore, since the transmission/disequilibrium test (TDT) follows transmission of alleles from heteozygous parents, power will be dictated by the heterozygosity of the alleles, which is impossible to know prior to the analysis of the data.

Hardy-Weinberg equilibrium will be assessed for all SNPs using control subjects as well as unaffected parents of probands. Minor allele frequencies (MAFs) and Linkage disequilibrium plots and coefficients (D') will be generated using Haploview, version 4.2[24]. Case-control analyses will be performed using multiplicative, additive, and dominant genetic models and tested for using Armitage's test for trend[25,26]. Family-based association testing (FBAT) and Haplotype-based association testing will be performed using the FBAT software

from Harvard's Department of Biostatistics[27]. The transmission disequilibrium test will be used to assess association of alleles at each marker with clefting with the use of the PLINK software, version 1.07[28].

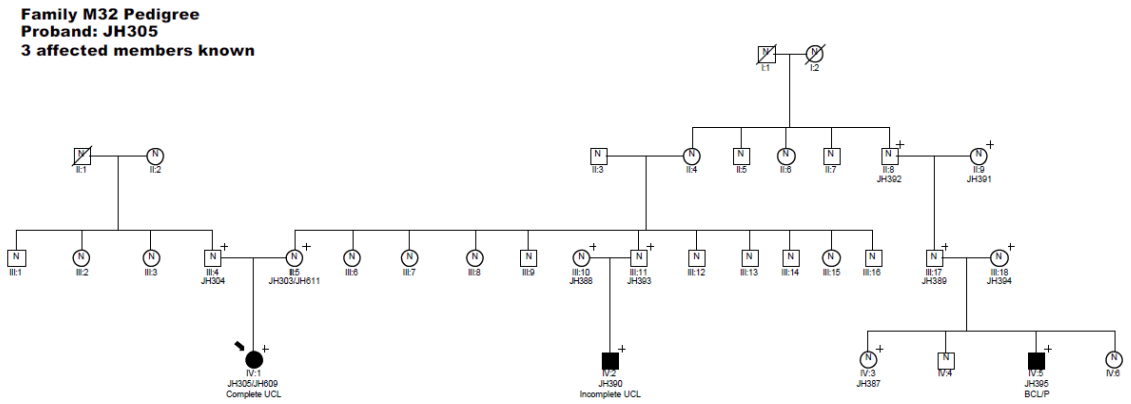
In order to conduct whole-exome analysis on a trait that demonstrates dominant inheritance with incomplete penetrance, it is optimal to select affected family members that are far removed from one another and thus share as little of the genome as possible. We have two families in which we have collected DNA from three affected family members that are sufficiently separated. Through this study design, we will optimize the likelihood of finding a causative mutation in these individuals. The percentage of genome these individuals share is as follows:

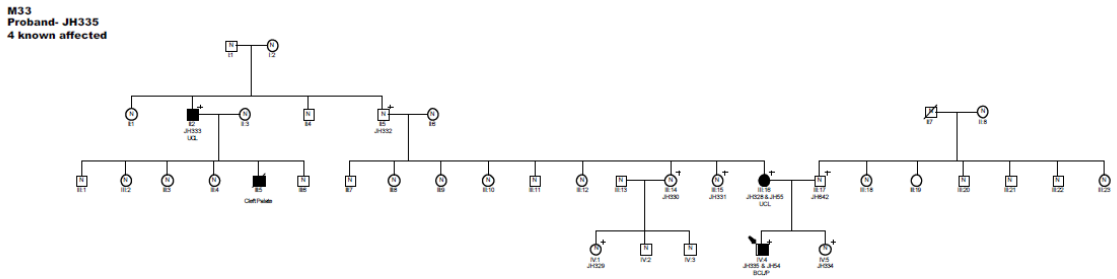
M32:

	JH305	JH390	JH395
JH305	X	12.5%	1.6%
JH390	X	X	3.1%
JH395	X	X	X

M33:

	JH328	JH333	JH335
JH328	X	25%	50%
JH333	X	X	12.5%
JH335	X	X	X





C. Study Procedure

Blood-drawing through venipuncture is the only procedure associated with the study. All children evaluated at the cleft clinic have on-going medical care, but their association with the study ends once blood is collected and family history is obtained. No more than 7.5 mL of blood are obtained from any one study patient.

D. Study Drugs N/A

E. Medical Device N/A

F. Study Questionnaires

A family cleft history and thorough history to rule out syndromic cleft lip and cleft palate as well as potential environmental causes (e.g. particular medications during pregnancy) are performed by the recruiting physician.

G. Study Subjects

Affected children (non-syndromic cleft lip with or without cleft palate) presenting to clinic and their primary family members (mother, father, grandparents, siblings) are recruited into the study. Additionally, affected relatives and their unaffected family members are recruited whenever possible. The majority of affected cleft patients presenting to the cleft clinic are in the pediatric age group, as most adults affected by cleft abnormalities have already undergone corrective surgery and do not regularly attend clinic. No children under 6 months of age are recruited.

H. Recruitment of Subjects

Affected children and their family members are recruited through the cleft clinic at Hospital Escuela in Tegucigalpa, Honduras by Honduran physicians and medical staff. Radio advertisements throughout the country are used to inform the population about the clinic's location and times of operation.

I. Confidentiality of Study Data

All data obtained will be kept confidential. Each participant will receive a study number without identifying information, such as name or birthday. Patient blood samples and DNA will be tracked with this code, and pedigrees will be generated using these codes as well. Research records will be kept in locked paper files and password protected computers, and records will only be accessible to authorized research staff or institutional personnel for routine audits. Study participants will not be informed of results of paternity testing or other genetic testing.

J. Potential Conflict of Interest

There are no conflicts of interest.

K. Location of the Study

Patients will be recruited in an orofacial clefting clinic in the Hospital Escuela, in Tegucigalpa, Honduras.

L. Potential Risks

Venipuncture is associated with some risks, such as local bruising, pain, bleeding, and infection at the puncture site. Standard safety precautions will be taken (i.e. wearing gloves, cleaning area with alcohol prior to puncture, placing pressure on wound after needle is extracted) to minimize these risks and blood draws will only be performed by experienced research staff. Loss of confidentiality is a risk inherent in genetics research. To minimize risks, each participant will have a study identification code stripped of identifying information that will be used to track DNA, blood, and pedigrees. All study documentation will be kept in password protected computers or locked paper files, and only authorized research personnel or institutional personnel performing routine audits will be allowed access. Patients will not be informed of the results of genetic testing.

M. Potential BenefIts

Patients receive no direct benefit for participating in the study. Any genetic markers found may assist in identifying future family groups at risk for cleft abnormalities and may provide the basis for future genetic counseling.

N. Alternative Therapies N/A

0. Compensation to Subjects

Families will be compensated approximated $5-$10 USD for travel to the clinic on the day of blood drawing depending on geographic distance traveled. These funds will be provided by the Honduran Medical Institute, Inc. in the form of Honduran Lempiras at the conclusion of the patient/family interview and venipuncture.

P. Costs to Subjects N/A

Q. Minors as Research Subjects

This study requires the participation of children. The majority of affected cleft patients presenting to the cleft clinic are in the pediatric age group, as most adults affected by cleft abnormalities have already undergone corrective surgery and do not regularly attend clinic. Informed permission from a parent or guardian will be obtained from young study participants who lack the maturity to provide assent. Children under the age of 6 months will not be eligible to participate in the study. Standard safety precautions will be employed and discomfort minimized by waiting until patients are under anesthesia to perform blood draws whenever possible. Blood draws are standard for assessments independent of this research proposal in clinic and are therefore presumed to be a minimal risk. No more than 7.5 mL of blood will be taken from any subject.

R. Radiation or Radioactive Substances N/A

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