A GENOME-WIDE LINKAGE DISEQUILIBRIUM ANALYSIS OF LOCI ASSOCIATING WITH NON-SYNDROMIC CLEFT LIP AND PALATE IN HONDURANS

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A. Study Proposal and Rationale

Cleft lip with or without cleft palate (CLP) and isolated cleft palate (CPI) are common congenital malformations, occurring in roughly 1.5-2 per 1,000 Caucasian births, with higher prevalence rates in Hispanic, Asian, and Native American populations.[3] These abnormalities have been associated with genetic and environmental causes such as maternal smoking and folate deficiency, as well as use of steroids, valproic acid, phenytoin, and methotrexate during pregnancy. Infants have increased risk of death secondary to prematurity, pneumonia, aspiration and sepsis, and adults have been shown to have increased risk of cardiac disease, suicide, epilepsy, and some cancers.[4]

CLP and CPI occur via different mechanisms and at different times during embryogenesis and are likely due to different genetic mechanisms, in contrast to environmental causes which contribute similarly to both types. There is a 2-5% increased risk for CLP and CPI in the offspring of affected individuals, with greater concordance in monozygotic than dizygotic twins and an increased relative risk of abnormalities in siblings of affected family members[8]. CLP and CPI may present as isolated malformations, but 4.3-63.4% of cases occur with congenital defects of other organ systems as part of over 400 identified syndromes.[4] The risk of syndromic cleft presentation is increased in individuals with CPI, with up to 50% of CPI individuals presenting with other abnormalities compared to 30% of those affected by CLP.[5 15] These syndromic cases often present with mental retardation and chromosomal abnormalities and generally follow Mendelian patterns of inheritance.[6] Non-syndromic forms of CLP demonstrate more complex, indeterminate inheritance patterns, with reduced penetrance and only 33% of affected patients reporting positive family history.[7]

The purpose of this study is to investigate the genetic basis of familial cases of non-syndromic CLP in a Honduran population. Identification of contributing genetic factors will aid genetic counseling, and may have future implications in the prevention and treatment of clinical disease. The genetics of CLP and CPI have been studied extensively, with multiple candidate genetic loci identified in many different populations. Candidate loci and genes include: 1q32 (IRF6), 2p13 (TGFA), 2q32-35 (Sumo1), 3p25, 4p16 (MSX1), 6q23-25, 8p21, 8q23, 11q23 (PVRL1), 12p11, 14q21-24 (TGFB3), 17q21 (RARA, CLF1), 18q21, 19q13 (BCL3), and 20q13.[1,8,9,10,11,12,14,15] However, no studies have been conducted to investigate the genetics of non-syndromic CLP in Hondurans, despite the increased prevalence of cleft abnormalities as compared to other populations. Roughly 90% of the population is Mestizo (Spanish and Native American) and remains fairly genetically homogenous. Because the prevalence of affected individuals is increased, there is potential for identification of common responsible mutations.

Being a trait of complex genetic and environmental origin, genome-wide linkage disequilibrium analysis will allow for potential discovery of new linkage and associations between multiple genes and the CLP or CPI phenotypes in the Honduran population. Identified genetic loci can then be mapped more finely to determine as precisely as possible what areas of the genome segregate with the clefting phenotype. Previous studies have been successful via this technique in identifying loci that associate with the clefting phenotype.[9,11,12,14]

B. Study Design

This study proposes a genome-wide linkage disequilibrium analysis of 70 nuclear families with two or more members affected by nonsyndromic CLP and CLI. Restricting analysis to families with two or more affected individuals helps to minimize the environmental contribution of sporadically arising disease and simultaneously increases the power of transmission/disequilibrium test (TDT) analysis. Blood samples and data from these families have already been collected for an ongoing, IRB approved study investigating associations between the clefting phenotype and SNP markers on three different genes. Pilot studies with these genes have not been powerful enough to yield significant associations: genome-wide analysis will allow for the identification of additional associated loci and potentially provide enough power to find association with the previously studied genes.

Families will be recruited for the study when patients present to the cleft clinic at Hospital Escuela, a public hospital in Tegucigalpa, Honduras. Patients will be screened by clinic staff for syndromic markers as well as potential environmental causes for disease (through history and physical exam), and included in the study only if they have nonsyndromic CLP or CPI and at least two affected family members. The type and laterality of the cleft as well as palatal involvement are to be noted during the complete physical exam by an examining physician. Pedigrees will then be digitally catalogued and blood samples procured from both affected and unaffected family members for DNA analysis through venipuncture. Blood draws are not performed for children scheduled for cleft repair until the day of surgery when under anesthesia in order to minimize psychological trauma. Blood is sent to Columbia University Medical Center via Federal Express, where DNA is extracted using a Qiagen Flexigene DNA Kit.

Affected members and their parents will be screened for familial associations using 38 microsatellite markers pre-selected for the earlier IRB study in order to minimize weakening of associations and linkage analysis. Families will not be informed of the results of genetic analysis, including familial association testing results.

C. Statistical Analysis

A genome-wide linkage disequilibrium analysis examines for co-segregation genetic marker alleles with a disease phenotype as well as genetic linkage. The further a genetic

marker is from genetic loci correlating with disease phenotype, the more likely they are to recombine and be transmitted randomly. Roughly 400 short tandem repeat polymorphic genetic markers spaced out at approximately 10-20 centiMorgans (cM) should be adequate to cover the entirety of the genome. However, isolated linkage studies lose power in genetically complex diseases where genetic heterogeneity interferes with development of a genetic model based on linkage to one gene. Linkage disequilibrium studies allow for measurement of linkage and association by examining for the non-random association of marker alleles near the loci, not just linked markers. The transmission/disequilibrium test (TDT) follows transmission of alleles from heterozygous parents at a locus by measuring deviation from the expected ratio of 0.5 for randomly associated marker alleles.[14]

Genotype data from the collected markers will be analyzed using the sib-TDT (S-TDT) method, which permits marker data from proband siblings to be used when data from both parents is unavailable. Due to socioeconomic limitations in Honduras, it is frequently difficult to obtain blood samples for both parents of a proband: often one must stay home while the other travels to clinic, and it is not uncommon to find access to one parent impossible.

In order to follow transmission of marker alleles to offspring, the “minimum” acceptable family structure for the TDT test is genotype data from one parent heterozygous for the marker as well as an affected offspring. This is in contrast to the S-TDT test, which ignores parental genotype data in favor of data from the unaffected sibling(s) of the affected offspring. The minimal acceptable family structure for this arrangement is two offspring, one affected and one unaffected, with different marker phenotypes. These two test types can be combined to allow for analysis of multiple available different family structures in one statistical process.[15]

D. 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 10 mL of blood are obtained from any one study patient.

D. Study Drugs and Devices

Not applicable.

E. 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.

F. 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. No children under 6 months of age are recruited.

G. Recruitment

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.

H. Confidentiality of 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.

I. 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.

J. 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.

K. Compensation

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.

L. 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 10 mL of blood will be taken from any subject.

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