A GENOME-WIDE SCAN FOR LOCI PREDISPOSING TO NON-SYNDROMIC CLEFT LIP WITH OR WITHOUT CLEFT PALATE IN A HONDURAN POPULATION

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A. Study Proposal and Rationale

Cleft lip with or without cleft palate (CL/P) and isolated cleft palate (CPI) are some of the most 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 (up to 1/500), and lower rates in African populations (1/2,000).[3] Clefting abnormalities are associated with increased morbidity and mortality in infancy, childhood, and adulthood. 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.[2]

Clefting abnormalities are associated with both environmental and genetic causes. The genetic basis for cleft lip and palate is supported by a 2-5% increased risk for offspring of affected individuals, greater concordance in monozygotic than dizygotic twins, and increased relative risk of abnormalities in siblings of affected family members. Additionally, both CL/P and CPI have been linked to environmental factors, such as maternal smoking, folate deficiency, and steroid, valproic acid, phenytoin, and methotrexate use during pregnancy. While the environmental contributions to CL/P and CPI are similar, their genetic causes most likely differ, which is not surprising given that CL/P and CPI form by different mechanisms and at different times during embryogenesis.[8]

While CL/P and CPI may present as isolated malformations, 4.3-63.4% of cases arise along with congenital defects of other organ systems as part of over 400 identified syndromes.[10] Additionally, some epidemiologic studies have demonstrated that the risk of syndromic cleft presentation is increased in individuals with CPI, with up to 50% CPI individuals presenting with other abnormalities compared to 30% of those affected by CL/P.[4,7] These syndromic cases are often marked by mental retardation and chromosomal abnormalities, and seem to follow Mendelian patterns of inheritance.[8] In contrast, nonsyndromic forms of CL/P demonstrate more complex inheritance patterns, with no recognizable mode of inheritance, reduced penetrance, and only 33% of affected patients reporting positive family history.[15] Therefore, it is important to distinguish between syndromic and nonsyndromic cases,[11] as well as CL/P and CPI, in the study of genetic risk factors.

Previous studies have suggested that CL/P results from the interaction of 3-14 genes, with each gene contributing a different relative risk for the abnormality. The genetics of CL/P 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.[1,8,9,12,13,14,16,17] However, to date, no studies have been conducted to investigate the genetics of CL/P in the Honduran population. Roughly 90% of its members are Mestizo (Spanish and Native American), and the prevalence of cleft abnormalities is increased compared to other populations (likely due to its Native American roots). Because the number of affected individuals in the population is increased, and because the population is homogenous, the effects of specific genes and alleles are likely to be enriched, and genetic analysis in this population may yield identification of particular loci associated with the cleft phenotype for this population.

Because CL/P is a complex trait, with multiple genes likely involved, genome-wide linkage analysis in which cosegregation of alleles as genetic markers with the disease phenotype within and across families may reveal new regions of the genome responsible the clefting phenotype in the Honduran population. Once these areas have been identified, the genetic loci associated with disease can be further narrowed using association mapping of areas showing evidence of linkage on the genome-wide scan with more closely spaced markers. This technique has been used in previous studies which have been successful in identifying new loci of interest.[9,13,14,16]

Identification of contributing genetic factors will allow for genetic counseling, as well as assist in identifying families at risk so that environmental factors may be manipulated to prevent and treat clinical disease.[8]

B. Study Design and Statistical Analysis

We propose a genome-wide linkage analysis of 50 nuclear families with two or more members affected by nonsyndromic cleft lip and cleft palate. 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. However, genome-wide analysis will allow for the identification of additional associated loci. The environmental contribution to the phenotype has been minimized by restricting analysis to non-sporadic families.

Patients presenting to the cleft clinic at Hospital Escuela, Honduras' largest public hospital, in Tegucigalpa and their families are recruited for the study. Upon presentation, patients are screened by clinic staff for syndromic markers (both through history and physical exam), and included in the study only if they have nonsyndromic CL/P and at least two affected family members. Additionally, questionnaires are provided to patients' mothers to exclude use of medications associated with clefting phenotypes during pregnancy. The examining physician notes the type and laterality of the cleft as well as palatal involvement, and performs a complete physical exam. An extensive family history is obtained to create pedigrees, which are digitally catalogued using Cyrillic 3 (a pedigree drawing program), and blood samples are procured from both affected and unaffected family members for DNA analysis through venipuncture. For children in whom reconstructive surgery is planned, blood draws are not performed until the day of surgery when the child is under anesthesia. Blood is sent to Columbia University

Medical Center via Federal Express, where DNA is extracted using a Qiagen Flexigene DNA Kit.

Of note: the cleft clinic is a component of a three year training program, funded by the Honduran Medical Institute, Inc., a nonprofit organization dedicated to the medical and surgical care of Honduran children with facial deformities, the Honduran Ministry of Health, The University of Honduras, and Hospital Escuela, which trains graduating residents in the medical and surgical care of children born with cleft abnormalities.

Because nonpaternity may weaken linkage associations, affected members and their parents will be screened for familial associations using 10 microsatelitte markers. Families in which familial associations are found to be erroneous will be excluded from genome-wide linkage analysis to minimize error and maximize linkage effects.

Genome-wide linkage analysis will be performed on remaining families using 400 total markers averaging 10cM apart across each chromosome. Genotype data from these markers will be analyzed using the evidential paradigm,[5,6] which separates error probabilities from evidence. In this model, probability of error is used for study planning but not in final analysis; rather, evidence of linkage is calculated from available data, and supported by LOD scores. The strength of evidence (k) necessary to reach a conclusion (either in favor of or against linkage) is determined along with expected penetrance for both recessive and dominant inheritance models, and the probability of weak (W) or misleading (M) evidence is calculated prior to analyzing data. This paradigm suggests that for $\Theta = 0.1$ (true linkage), k=32, and 0.8 penetrance, and n=50 families, the probability of wrong or misleading evidence is < 5% for both dominant and recessive models of inheritance.[5,6] Therefore, roughly 50 families should provide enough power to demonstrate linkage when it is present. An LOD score will be calculated for each marker, and loci with a LOD score > 3.3 (strong evidence for linkage) will be evaluated using second-stage mapping with more densely spaced markers (averaging 5cM apart). LOD scores <3.3 but >2.0 will also be considered for second stage mapping.

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 7.5mL 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 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 unaffected 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. To minimize these risks, 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) and blood draws will only be performed by experienced research staff.

Particularly because this research involves genetics information, loss of confidentiality is another risk. To minimize risks, each participant are given a study identification code without 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 genetics testing.

J. Potential Benefits

Patients receive no direct benefit for participating in the study. If genetic markers are found, they 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 (depending on region from which patients are traveling) for travel to the clinic on the day of blood drawing. These funds will be provided by the Honduran Medical Institute, Inc. Payment will be offered 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. Most affected cleft patients presenting to the cleft clinic are in the pediatric age group, as most older adults affected by cleft abnormalities have already undergone a corrective procedure by the time they are identified for study participation. Care will be taken to minimize discomfort and risks to these patients, both by adhering to standard safety precautions and waiting until patients are under anesthesia to perform blood draws whenever possible. No more than 7.5 mL of blood will be taken from any subject. This is considered minimal risk, as blood drawing is a component of clinical care for these patients independent of study participation. Children under the age of 6 months will not be eligible to participate in the study.

Because cleft patients presenting to the clinic are often very young (0-5 years of age), some study participants will be too young or lack the maturity to provide assent. In these cases, the informed permission from a parent or guardian will be obtained.

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