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1) A Genome-Wide Linkage Analysis of Loci Associating with Non-Syndromic Cleft Lip With or Without Cleft Palate in a Honduran Population

2) IRF6 Gene Mutations and the Van der Woude Syndrome (VWS) in the Honduran Population

A. Study Proposal and Rationale – For both Nonsyndromic and VWS study

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 greater prevalence in Hispanic Asian, and Native American populations (up to 1 per 500), and lower prevalence in African populations (1 in 2,000).[1] 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.[2] Adults have been shown to have increased risk of cardiac disease, suicide, epilepsy, and some cancers.[2]

CLP and CPI are associated with both environmental and genetic causes. Environmental factors include maternal smoking and folate deficiency, as well as the use of steroids, valproic acid, phenytoin, and methotrexate during pregnancy.[3] The genetic basis of cleft lip and palate is supported by a 2-5% increased risk of CLP and CPI in the offspring of affected individuals, greater concordance in monozygotic than dizygotic twins, and increased relative risk of abnormalities in siblings of affected family members.[3] Environmental factors contribute similarly to both CLP and CPI, whereas genetic causes of CLP and CPI differ due to the fact that CLP and CPI form by different mechanisms and at different times during embryogenesis.[3]

Although CLP and CPI may present as isolated malformations, 4.3-63.4% of cases occur along with congenital defects of other organ systems as part of over 400 identified syndromes.[4] In addition, epidemiologic studies have demonstrated that the risk of syndromic cleft presentation is increased in individuals with CPI, with up to 50% of CPI individuals with a syndromic presentation (CPI with other abnormalities) compared to 30% of those affected by CLP.[5, 6] Syndromic cases of CLP and CPI often present with mental retardation and chromosomal abnormalities, and generally follow Mendelian patterns of inheritance.[3] Nonsyndromic forms of CLP demonstrate more complex inheritance patterns, with indeterminate inheritance patterns, reduced penetrance, and only 33% of affected patients reporting a positive family history.[7] Therefore, in the study of genetic risk factors, it is important to distinguish between syndromic and nonsyndromic cases of CLP and CPI.[8]

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.[3, 7, 9-14] Despite the increased prevalence of cleft abnormalities in the Honduran population as compared to other populations, only one study has been conducted to investigate the genetics of nonsyndromic CLP

in Hondurans.[15] The Honduran population is roughly 90% Mestizo (Spanish and Native American) and remains fairly genetically homogenous. The prevalence of cleft abnormalities is increased compared to other populations (likely due to its Native American roots). Due to the fact that the prevalence of affected individuals is increased, and the population is homogenous, the effects of specific genes and alleles are likely to be enriched, and genetic analysis in this population could yield identification of particular loci associated with the clefting phenotype.

CLP is a complex trait, likely with multiple genetic factors involved, a genome-wide linkage disequilibrium analysis using cosegregation of alleles as genetic markers with disease phenotype within and across families will allow for potential discovery of new linkage and associations between multiple genes and the CLP or CPI phenotypes in the Honduran population. 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. Previous studies have been successful using this technique in identifying new loci of interest that associate with the clefting phenotype.[7, 10, 12, 13]

In a recently published study by Diercks et al. an association was found between interferon regulatory factor 6 (IRF6) and nonsyndromic CLP in a Honduran population.[15] Family-based joint linkage and association analysis was used to evaluate five single nucleotide polymorphisms (SNPs) in and around IRF6. These five SNPs were previously reported to be associated with nonsyndromic CLP and were tested for association with nonsyndromic CLP in 59 families with at least two members affected by clefting and at least one member with confirmed nonsyndromic CLP (with a total of 276 affected and unaffected Honduran individuals). The five candidate SNPs in the IRF6 gene were rs7543025, rs2357075, rs1856161, rs2235371, and rs2235377. These candidate SNPs showed a strong association with nonsyndromic CLP in the Filipino population.[14] In the study by Diercks et al. the SNP rs2357075 was excluded because it was found to be monomorphic in the study population.[15] The other four SNPs were evaluated using linkage and association analyses. Support for linkage was found for three SNPs – rs1856161, rs2235371, and rs2235377. This was the first study to demonstrate that three candidate SNPs within IRF6 are significantly associated with nonsyndromic CLP in the Honduran population.

The purpose of this study is to investigate the genetic basis of familial cases of nonsyndromic CLP in the Honduran population. Diercks et al. identified the IRF6 gene is associated with nonsyndromic CLP in the Honduran puplation.[15] Detailed studies with a dense coverage of SNPs are needed in the Honduran population to further evaluate the association between IRF6 and nonsyndromic CLP and identify the putative variants within the IRF6 gene. Identification of contributing genetic factors will aid in genetic counseling, as well as assist in identifying families at risk in order to manipulate environmental factors to prevent and treat clinical disease.[3]

Secondary Study

IRF6 Gene Mutations and Van der Woude Syndrome in the Honduran Population

While over 70% of CLP are non-syndromic, over 300 syndromes have CLP as one of the features.[16] The highest number of syndromic disorders are caused by Van der Woude syndrome (VWS), which accounts for 2% of all cleft cases, affecting about 1 in 100,000-200,000 individuals.[17] VWS is a rare developmental, congenital malformation with autosomal dominant

inheritance, high penetrance and variable expressivity.[18] The penetrance has been reported to be 80%, yet may be close to 100% if supposedly unaffected carriers are examined for minor expressions of VWS.[19] The cardinal signs of VWS are lower lip pits, CLP, and CPI. Hypodontia (absent teeth) has also been increasingly recognized as a frequently associated anomaly. Other milder expressions of the phenotype include verrucous eminence in the lower lip, submucous cleft palate and bifid uvula.

Due to the similarity in the clefting phenotype, VWS could serve as a simpler model to study the more complex nonsyndromic forms of orofacial clefts.[20] The variability of expression within family members carrying the same mutation suggests multifactorial influences similar to nonsyndromic cases.

The orofacial anomalies present in VWS are caused by an abnormal fusion of the palate and lips at 30-50 days postconception. Several mutations in the IRF6 gene have been found in VWS families, suggesting that this gene is the primary locus. The gene encoding IRF6 is located in the critical region for the VWS locus at chromosome 1q32-q41.[21, 22] The IRF6 gene has been shown to regulate fetal craniofacial development in mice. Kondo et al. published evidence of expression analyses that showed high levels of IRF6 mRNA along the medial edge of the fusing palate, tooth buds, hair follicles, genitalia and skin.[23] Linkage studies have shown that a second modifying gene mapped to chromosome 17p11.2-p11.1 may enhance the probability of CP in an individual carrying the VWS gene at 1q32-q41.[24] A second chromosomal locus at 1p34 has also been identified.[25]

Former studies were performed in a Northern European population.[23] The genetics of VWS have not been studied in the Honduran population. The goal of this study is to determine if VWS in Honduras represents a genetically distinct population. For instance, the study will search for similarities or differences between the genetics of VWS in Honduras and other populations.

B. Study Design – VWS study

This study proposes a genome-wide linkage analysis of four families with two or more family members affected by VWS. Restricting analysis to families with two or more affected individuals helps to minimize the environmental contribution of sporadically arising disease and simultaneously increased the power of transmission/disequilibrium (TDT) analysis. Blood samples and data from these families have already been collected for an ongoing, IRB approved study investigating associated between the clefting phenotype and various candidate SNPs.

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 markers of VWS (cleft lip, cleft palate, hypodontia, lower-lip pits) through a history and physical exam. 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. An extensive family history is obtained to create pedigrees, which are digitally catalouged using Cyrillic 3 (a pedigree drawing program). Blood samples are procured from both affected an unaffected family

members for DNA analysis through venipuncture. Blood draws are not performed for children scheduled for cleft repair until the day of surgery while 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 Fexigene DNA kit.

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

C. Statistical Analysis – VWS study

A genome-wide linkage 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. 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 heteozygous parents at a locus by measuring deviation from the expected ratio of 0.5 for randomly associated markers alleles.[26]

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. It is not uncommon to find access to one parent impossible.

In order to follow transmission of marker alleles to offspring, the minimal 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 structure in one statistical process.[27]

If our study confirms the findings of prior studies, showing an association between VWS adn the IRF6 gene, mutation analysis will be performed by sequencing the IRF6 gene. First, primers will be developed for the nine exons on IRF6. Seven of the nine exons are coding exons, while the other two are non-coding exons. Using the primers, standard PCR will be performed to amplify all nine exons. The amplified products will be purified and directly sequenced by the company MacroGenUSA. The sequence will be analyzed using the computer program PolyPhred. Statistical significance of mutation location will be calculated with the Fisher's exact test using the assumption of equal probability for a mutation at each residue.

D. Study Procedure – VWS study

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 is obtained from any one study patient.

E. Study Drugs and Devices – VWS study

Not applicable.

F. Study Questionnaires – VWS study

The recruiting physician performs a family cleft history and thorough history to rule out potential environmental causes (such as particular medications during pregnancy).

G. Study Subjects – VWS study

Affected children with VWS 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.

H. Recruitment – VWS study

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 Data – VWS study

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. Records will only be accessible to authorized research staff or institutional personnel for routine audits. Study participants will not be infomred of results of paternity testing or other genetic testing results.

J. Potential Risks – VWS study

Venipucture 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 (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.

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 infomred of the results of genetic testing.

K. Potential Benefits – VWS study

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.

L. Compensation – VWS study

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

M. Minors as Research Subjects – VWS study

This study requires the participation of children. Most affected cleft patients presenting to the cleft clinic are in the pediatric age group, as the majority of older 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. No more than 10 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.

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