

5-2010

Analysis of Variation in Clubfoot Candidate Genes

Audrey R. Ester

Follow this and additional works at: http://digitalcommons.library.tmc.edu/utgsbs_dissertations



Part of the [Genetics Commons](#), and the [Molecular Genetics Commons](#)

Recommended Citation

Ester, Audrey R., "Analysis of Variation in Clubfoot Candidate Genes" (2010). *UT GSBS Dissertations and Theses (Open Access)*. 7.
http://digitalcommons.library.tmc.edu/utgsbs_dissertations/7

This Dissertation (PhD) is brought to you for free and open access by the Graduate School of Biomedical Sciences at DigitalCommons@TMC. It has been accepted for inclusion in UT GSBS Dissertations and Theses (Open Access) by an authorized administrator of DigitalCommons@TMC. For more information, please contact laurel.sanders@library.tmc.edu.

Analysis of Variation in Clubfoot

Candidate Genes

by

Audrey R. Ester, BS

APPROVED:

Supervisory Professor Jacqueline T. Hecht, PhD

Gilbert Cote, PhD

Hope Northrup, MD

James Hixson, PhD

Karen Storthz, PhD

APPROVED:

Dean, The University of Texas
Health Science Center at Houston
Graduate School of Biomedical Sciences

Analysis of Variation in Clubfoot Candidate Genes

A DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M. D. Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment
of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

by

Audrey R. Ester, BS

Houston, Texas
May, 2010

Dedication

I would like to dedicate this work to my parents who have always had faith in me and supported me throughout my graduate career.

Copyright © 2010 Audrey Ester. All rights reserved.

Acknowledgements

First and foremost I must thank Dr. Hecht for her guidance and mentorship. She spent many, many hours editing my writing, making suggestions to my experiments, and finding me help when I needed it. She has a better student and a better scientist. Dr. Gil Cote also provided much needed advice for both scientific and personal adversities. His dedication to our program and to HMG students has been a huge support system, and is greatly appreciated.

I would like to thank my family for always supporting me in my endeavors, being a shoulder to cry on, and providing a voice of encouragement. Thank you so much for everything you have done for me.

My friends provided the needed distraction and advice during graduate school, and I would not have survived without you. My labmates, Brett, Katelyn, and Tom kept me sane during my work, laughed at my mistakes, and then helped me to fix them. Katelyn, feel free to call with any problems, and please no conference calls with singing... Many thanks to Kim, with whom “Audrey and Kim Night” was developed, and I am ever so grateful that we did not live up to your mother’s prediction of “Audrey and Kim do jail”!

I must also thank fellow lab members who have always been willing to help me with my lab work, go on coffee runs, and make suggestions on presentations and publications. Specifically, I would like to thank Dr. Karen Posey, who received almost daily questions about protocols I was unfamiliar with and the following discussions when I did not know how to interpret the results. Finally I must thank all of the GSBS staff who has helped me in my career as a graduate student. Thank you for the advice and hard work.

Analysis of Variation in Clubfoot Candidate Genes

Audrey Ester

Advisor: Jacqueline T. Hecht, PhD

Isolated clubfoot, a common birth defect occurring in more than 135,000 livebirths worldwide each year, is associated with significant health care and financial burdens. Clubfoot is defined by forefoot adduction, hindfoot varus, midfoot cavus and hindfoot equinus. Isolated clubfoot, which is the focus of these studies, is distinct from syndromic clubfoot because there are no other associated malformations. Population, family, twin and segregation analysis studies provide evidence that genetic and environmental factors play an etiologic role in isolated clubfoot. *The studies described in this thesis were performed to define the role of genetic variation in isolated clubfoot.* Interrogation of a deletion region associated with syndromic clubfoot, suggested that *CASP8* and *CASP10*, two apoptotic genes, play a role in isolated clubfoot. To explore the role of apoptotic genes in clubfoot, SNPs spanning genes involved in the apoptotic pathway in the six chromosomal deletion regions, and limb patterning genes, *HOXD* and *HOXA*, were interrogated. SNPs in mitochondrial mediated apoptotic genes and several SNPs in *HOXA* and *HOXD* genes were modestly associated with clubfoot with the most significant SNP, rs3801776, located in the basal promoter of *HOXA9*. Several significant associations were found with SNPs in *NFAT2* and *TNIP2*. Significant gene interactions were detected between SNPs in *HOX* and apoptotic genes. These findings suggest a model for clubfoot in which variation in one gene is not sufficient to cause the malformation but requires variation several genes to perturb protein expression sufficiently to alter muscle and foot development. **These results significantly impact our knowledge base by delineating underlying mechanisms causing clubfoot.**

Table of Content

Dedication	iii
Acknowledgements	iv
Table of Content.....	vi
Chapter 1: Background.....	1
Introduction to Clubfoot	2
Musculature and tendons of clubfeet	2
Treatment of clubfoot	5
Conservative Treatment	5
Operative Treatment	6
Complications of Treatment	6
Treatment Innovations	7
Normal foot development.....	7
FGF signaling.....	9
Sonic Hedgehog and <i>GLI3</i> signaling	9
Homeobox (<i>HOX</i>) Genes.....	10
Retinoic Acid	10
Apoptosis	11
Causes of Clubfoot.....	13
Genetic Mapping.....	15
Previous Genetic Studies	16
Significance	19

Chapter 2: Materials and Methods	21
Study Population.....	22
Samples.....	22
Gene Selection	23
Genotyping.....	23
Statistical Analyses	24
Sequencing.....	25
Transcription Factor Binding Prediction	25
Chapter 3: Mitochondrial Mediated Apoptotic Pathway and Clubfoot.....	26
Background of Apoptosis and clubfoot	27
Deletion Regions Associated with Clubfoot.....	27
Fine Mapping of Chromosome 2 Deletion Region.....	27
Apoptosis	28
Mitochondrial Mediated Apoptosis	28
Activator Caspases.....	28
BID.....	30
BCL-2	30
CASP9 and APAF-1	31
CASP3.....	31
Study Design.....	31
Results.....	32
Discussion.....	38
Chapter 4: Analysis of Candidate Genes in Clubfoot Deletion Regions.....	41

Introduction.....	42
Immunity Related Genes.....	43
Transcription Factor Genes.....	47
Engulfment of Apoptotic Body Genes.....	49
Other Apoptosis Related Genes.....	50
Methods.....	52
Sample Collection.....	52
DNA Extraction and Genotyping.....	53
Data Analysis.....	53
Results.....	53
Discussion.....	63
Chapter 5: HOX Transcription Factors and Clubfoot.....	68
HOX Genes.....	69
Study Design.....	70
Results.....	71
Discussion.....	78
Chapter 6. Conclusions and Future Studies.....	80
Conclusions.....	81
Future Studies.....	83
References.....	85
Appendix A: Mitochondrial mediated apoptosis supplemental tables.....	105
Appendix B: Clubfoot Chromosomal Deletion Region Apoptotic Genes Supplemental Tables.....	111

Appendix C: HOXA and HOXD Supplemental Tables 136

Chapter 1: Background

Introduction to Clubfoot

Foot deformities are common. One group results from flexible positional abnormalities and are easily treated with minor manipulations and is likely due to intrauterine constraint¹. The second group is rigid malformations referred to as talipes equinovarus, and is the only true clubfoot. This malformation involves the foot held in equinus (rigid downward position) and adducted (turned towards the midline of the body)². Clubfeet often have misshapen bones such as a flattened talus, the navicular is often vertical instead of horizontal, and cuneiforms are often stacked behind each other². The position of the clubfoot resembles an embryonic foot at the beginning of the second month, although during no stage of normal development is the navicular vertical². While the bones may be misshaped and aligned differently in clubfoot, it is unknown if this is the primary cause of clubfoot, or if it is secondary to abnormal musculature or tendon placement.

Musculature and tendons of clubfeet

Clubfeet are also characterized by hypoplasia of the calf muscles, which often persists throughout life; however there are conflicting studies about muscle placement, fiber density, and even muscle size³. Irani and Sherman examined eleven fetal clubfeet from 22 to 36 gestational weeks of age³. In one case, the cross-section of the calf muscle from the affected limb was larger than the unaffected limb, but the muscles appeared to be the same size bilaterally even though the limb appeared larger³. However, another affected foot showed muscle masses that corresponded to the unaffected foot, but each mass was much smaller than that of the control limb³. In general, Irani and Sherman concluded that muscles of clubfeet are underdeveloped and smaller when compared to unaffected limbs³. Because the

calf muscles have found to be hypoplastic, they have been the focus of several histological studies in clubfeet.

In 1977, Isaacs et al. investigated the types, number, size, and direction of muscle fibers in individuals with clubfeet. This study concluded that clubfoot calf muscles are “grossly abnormal”, with higher numbers of Type I fibers, and both types of muscle fibers were larger than controls⁴. Type I muscle fibers are also known as slow twitch muscle fibers and are redder in color because they have a higher myoglobin and oxygen content^{5,6}. These fibers are used in long-term activity because they resist fatigue, as opposed to fast twitch (Type II) muscle fibers, which are used during short bursts of activities, are more fatigable and can only receive energy from glycolysis^{5,6}. In addition to higher numbers of Type I muscle fibers, Isaacs et al. found evidence of denervation in calf muscles of clubfoot⁴. The larger muscle fibers imply that atrophy is not the reason for smaller muscles, but might be caused by a neuropathy⁴. The patients in this study were referred for study due to the presence of a rigid talipes equinovarus, but does not mention any other associated abnormalities⁴.

Fukuhara et al. performed histomorphometric and immunohistochemical studies on 16 fetal clubfeet and 27 normal feet (seven normal feet from unilateral patients and 20 feet from spontaneously aborted fetuses of the same age range as those affected)⁷. This study found that the bone malformations were secondary to ligament and collagen changes⁷. The tibialis posterior ligament was found to be enlarged, and the orientation of the collagen fibers was disrupted⁷. A syndromic clubfoot animal model was created in the 1960s by Drachman et al. through the paralysis of chick embryos⁸. These animals exhibit “jaw deformities, scoliosis, characteristic flexion contractures of the knee, ankle, and toe joints, with severe deformity of the articulating surfaces”⁸. Germiller et al. used this model to determine the muscle and

tendon sizes in this model compared to control chicks⁸. The total volume of muscles and soft tissue of the paralyzed chicks decreased as the dose of paralyzing drug increased⁸. In addition the tendons were significantly smaller with increasing paralysis and merged at the bottom of the calf⁸. This is in contrast to the size of the cross-sectional area of the tibial muscle, which was not correlated with the drug dose⁸. This suggests that in the drug-induced clubfoot model, muscle and tendon cross-sectional area were smaller than those of controls⁸. This supports data from previous studies that suggest that calf muscles in nonsyndromic clubfeet are abnormally small.

In 1998, a prospective study was performed by Loren et al. in which muscles of children with clubfoot were examined histologically, and further investigated if surgical intervention impacted these histopathological findings¹. About half of the patients with clubfeet showed abnormal muscle morphology¹. Twenty percent had fiber type disproportion, and 30% had fiber size variation (>3:1 ratio)¹. The clubfeet with abnormal muscle morphologies had an increased risk for clubfoot recurrence and a second operation (5.6 fold increase)¹.

Omeroglu et al. studied ten fetuses with clubfoot who also had spina bifida⁹. Spina bifida is often associated with deformities of the foot and ankle, which usually result from muscle imbalance or denervation. Of these foot deformities, clubfoot is the most common in spina bifida patients with “L3 and higher neurosegmental lesions”⁹. The muscle size of the control group was larger than that of the affected limbs, and there was a higher proportion of fibrosis in clubfoot limbs than those of control muscles⁹. This study concluded that the fibrosis was secondary to atrophy in the skeletal muscle tissue, and the clubfoot is most the result of muscle inactivity⁹. This is possibly due to the atrophy or the atrophy may occur because of the lack of muscle movement⁹.

A histological study of clubfoot in 2006 reported on 431 muscle specimens obtained from 68 patient surgeries. This study gave very different results with 86.3% having “no discernible pathology”¹⁰. Atrophy and an over-abundance of type I muscle fibers was found in 12.8% of the specimens¹⁰. This study was much larger than the Omeroglu study and was a better representation of the average clubfoot compared to normal musculature. Other case studies have reported an additional muscle coming out of the soleus muscle.¹¹⁻¹³ There have also been reports of a flexor digitorum accessorius longus muscle that is aberrant in children with clubfoot¹⁴⁻¹⁶. These case studies involve small numbers of clubfeet and from individual collection sites. In addition, many of these evaluated the “clubfoot deformity” but do not discriminate between idiopathic clubfoot and syndromic clubfoot, and so these clubfeet might have different musculature due to syndromes (such as a neuropathy). A large-scale comprehensive study in idiopathic clubfoot patients is needed to validate these findings.

Treatment of clubfoot

There are several methods of clubfoot treatment, including aggressive operative intervention, conservative manipulations of the feet, and a combination of the two methods¹⁷. Historically, operative treatments were the first choice among doctors, often performing a comprehensive treatment that uses surgery to correct all aspects of the deformity. However, a more conservative approach has been taken consisting of manipulation, casting, and bracing of the foot, followed by surgery if necessary¹⁷.

Conservative Treatment

The two most common conservative treatments are the Ponseti and Montpelier (French) methods, which both promote the progressive stretching of the muscles and tendons in an

attempt to forgo surgical soft tissue release¹⁸. The Ponseti method consists of serial castings that hold the foot in an overcorrected position, a tendonectomy if needed, and then braces that again hold the foot in an overcorrected position¹⁸. The Montpellier method requires much more intensive participation on the part of the physician and requires the patient to have long inpatient manipulations and “continuous passive motion”¹⁸. The Montpellier method is more expensive and not widely used outside of France¹⁸.

Operative Treatment

Operative treatments consist of individual surgeries to address each component of the clubfoot deformity, or can repair multiple components or the entire deformity. Many doctors who prefer to use an operative treatment accept that the end result is not a normal foot¹⁷. Posterior release is the most common surgery after conservative treatment, although more aggressive surgery may be necessary. This soft tissue release consists of releasing the Achilles tendon, as well as tendons restraining the ankle and subtalar joints¹⁸. If the clubfoot deformity is more extensive, it may require medial and lateral tendon releases as well¹⁸.

Complications of Treatment

The Ponseti method has a high rate of correction, but almost always requires an Achilles tenotomy¹⁸. Usually the only complications of the Ponseti method are related to slippage of the casts or failure to comply with bracing¹⁸. Undercorrection following surgery can be difficult because the scar tissue is less flexible and hinders further surgeries¹⁸. Overcorrection is harder to treat, and can even cause a “rocker bottom” foot¹⁸. The most common complication for all methods of treatment is recurrence of the clubfoot¹⁸. The Ponseti method treats recurrent clubfeet by recasting or rebracing until the deformity is

corrected and possible surgery if necessary¹⁸. Recurrence can also occur because of scar tissue following surgery preventing the stretching of muscles and tendons during bracing¹⁸. The Ilizarov technique is used almost exclusively for recurrent clubfeet, although long term success has not been reported¹⁷. The Ilizarov method uses an external, circular frame, and this method is often supplemented with other types of surgery as well.

Treatment Innovations

Botulinum toxin-A (Botox) has been used as a substitute for an Achilles tenotomy¹⁷. The results of one study are a disappointment as the injected infants showed no difference than those injected with a placebo with regard to further treatment¹⁷. However, additional studies have shown that Botox is an effective treatment and reduces the need for tendon releases^{19,20}. Surgery is still required in few cases, but Botox can still be used as a conservative treatment before resorting to tendon release²⁰.

In order to improve treatments for children with clubfoot, the extent of the tissues affected and the cause of the deformity need to be determined. In addition, correct classification and identification of the causes of clubfoot are needed.

Normal foot development

Limb formation is a complex process involving cell proliferation, patterning, and programmed cell death^{21,22}. The hind limb begins to develop as a small swelling during the fourth week of gestation, with morphogenesis being completed by the end of the eighth week²³. The legs, which develop in the midline in equinus should move to plantar grade by the twelfth week²⁴. Perturbations in any of the developmental processes may affect the final positioning, particularly those causing hypoplastic muscles or shortened tendons that are

universally associated with clubfoot^{1,7,11,14}. The hind limbs are composed of three basic segments: the stylopod (upper limb), the zeugopod (lower limb), and the autopod (foot). The expansion and differentiation of cells in the limb bud is regulated by many secreted signaling factors.

Signaling factors of normal foot development

Limb development involves both dorsal ventral patterning and proximal distal outgrowth, which requires an intricate expression and diffusion pattern of growth factor signaling²⁵. There are three major signaling centers that control these axes of development, the AER, the zone of polarizing activity (ZPA), and the ectoderm²⁶. The AER maintains the proximal distal outgrowth, the ZPA migrates behind the AER regulating the anterior-posterior axis, and the ectoderm controls the dorsal-ventral axis²⁶.

The emerging limb bud forms with the leading most edge of the outgrowth, known as the apical ectodermal ridge (AER)²⁶. The AER maintains proliferating cells and secretes factors that prevent cells from differentiating into cartilage²⁵. The AER is characterized by both proliferating cells and cells undergoing programmed cell death, and these have been shown to be in the same regions²⁶. One model theorizes that the longer cells remain in the AER, the more distal the cells are located when they differentiate²⁷. Another theory suggests that the fate of cells comprising the limb is predetermined and therefore time does not dictate the differentiation of cells²⁷. Outside of the AER, somites begins to proliferate and differentiate into muscle precursors²⁶.

These signaling centers secrete growth factors and other signals that diffuse through the limb bud, interacting and forming overlapping and diverging gradients²⁶. These signaling

regions are conserved throughout vertebrate development including fish²⁸. This interplay of secreted signaling factors is what leads to the distinct and normal limb²⁶.

FGF signaling

Limb development first begins with the expression of fibroblast growth factor (*FGF*) 8, which is expressed by the intermediate mesoderm in the trunk of the embryo²⁷. This first wave of FGF8 induces expression of *WNT-2B* and *-8C*, which in turn stimulates expression of *FGF10*²⁷. FGF10 signals a second wave of FGF8 expression in the AER²⁷, and in turn maintains expression of *FGF10*²⁷. The AER is controlled primarily by *FGFs*, including FGF2, 8, 9 and FGF receptor (FGFR) 2b, which are expressed constitutively throughout the AER, and *FGF4* and *17* are expressed in the posterior AER²⁷. Because of the expression pattern of *FGF4* and *17*, it is thought that these growth factors stimulate and maintain the ZPA²⁷.

Sonic Hedgehog and *GLI3* signaling

Sonic hedgehog (*SHH*) is a secreted signaling molecule that is interpreted through another molecule, *GLI3*²⁹. In limb development, *SHH* regulates the distribution of *GLI3*, and *GLI3* is in turn regulated by *SHH* through processing²⁹. *SHH* expression is restricted to the ZPA, and is thought to act as a morphogen by regulation other signaling factors depending on its concentration in specific tissues and regions²⁹. However, a double knockout of *SHH* and *GLI3* showed a restoration of ventral cell types that were missing in *SHH*^{-/-} mice, indicating that the extent of their functions is unknown²⁹.

Within the ZPA, *SHH* is responsible for the patterning of the digits²⁹. Ectopic expression of *SHH* causes polydactyly²⁹. However knockout of *GLI3* can produce synpolydactyly,

which suggests that *GLI3* may be involved in apoptosis and separation of digits²⁹. However, the knockouts of *SHH* or *GLI3* do not affect the distal portions of the limb, suggesting that the ZPA is involved only in the initial development of the autopod²⁹.

SHH also stimulates the expression of other genes involved in limb development including *HOXD* genes³⁰. *SHH* is a homolog of hedgehog (*HH*) in drosophila, and it has been shown to regulate gene expression of members of the bone morphogenic protein (*BMP*) family, which can stimulate apoptosis³⁰.

Homeobox (*HOX*) Genes

The *HOX* genes are transcription factors which regulate differentiation and patterning of cells^{31,32}. In vertebrates there are 39 *HOX* genes in four clusters, and each cluster is on a separate chromosome. The *HOX* clusters are regulated temporally and spatially by global promoters, noncoding RNAs, transcription factors, and other regulatory elements during embryogenesis^{31,33}. 5' genes of the *HOXA* and *HOXD* clusters (9-13) are expressed in the mesoderm and muscles of the developing limbs³⁴. The 5' genes are expressed later and more distally with *HOXA13* and *HOXD13* being expressed latest and in the distal autopod³⁵. *HOX* mutations lead abnormal limb development, and each of the 5' genes correlates with a missing limb segment³⁵. A deletion of *HOXA11* and *HOXD11* results in elimination of the zeugopod, but the deletion of *HOXA13* and *HOXD13* causes absence of the autopod³⁵.

Retinoic Acid

Retinoic acid (RA) is derived from vitamin A, which is necessary for normal embryo development²⁸. RA acts as a transcription factor binding to retinoic acid response elements (RAREs) that either activate or repress genes²⁸. High levels of retinoic acid can lead to birth

defects, but a deficiency of retinoic acid can prevent normal embryonic development²⁸. RA is postulated to be a morphogen, a molecule to which cells respond differently depending on its concentration²⁸. RA can stimulate SHH production and can also mimic a ZPA graft, leading to digit duplications³⁰. While the implantation of a RA bead in the limb causes polydactyly, there is no evidence that it plays a direct role in normal limb development²⁸. However, RA expression is required in the trunk of the embryo where the limb buds will form³⁶. This early expression is needed even though other factors will be expressed in the region such as SHH which also confer limb bud placement³⁶. Retinoic acid deficiency can lead to a wide array of malformations including cleft palate, unfused nasal passages, and syndactyly³⁶. These malformations reported result from a lack of apoptosis, suggesting that RA can induce/regulate apoptosis during embryonic development³⁶.

Apoptosis

Apoptosis or programmed cell death (PCD) is the process that a cell undergoes when it receives signal(s) indicating that the cell is no longer needed³⁷. Typically this is associated with the shaping of the limb including digits and other skeletal elements³⁷. Apoptosis occurs in many different regions of the developing limb, although some major regions of apoptosis have been named necrotic regions because their naming occurred before the discovery of PCD³⁷. A large region of apoptosis in the mesenchyme is known as the opaque patch, which regulates PCD in the skeleton of the zeugopod³⁷. Failure of apoptosis to occur in the opaque patch leads to fusion of the tibia and fibula³⁷.

There is also apoptosis in the interdigital mesoderm which results in the formation of separate fingers, and its absence can lead to a fusion of the fingers³⁷. During the outgrowth of the limb bud, apoptosis occurs in the AER, and if that apoptosis is inhibited, the AER

overgrows and results in polydactyly³⁷. Studies have also shown a role for apoptosis in joint formation and possibly guiding nerves through the developing limb²². In addition to these large regions of apoptosis, there is also PCD within the developing muscles and tendons²².

Muscle and Tendon Development

Muscles cells originate from the somatic mesoderm, produced through signaling factors *PAX3* and *MYOD*, and then migrate to the appropriate location under control of Homeobox gene, *LBX1*²². These muscles migrate to regions that correspond to the major delineation of the limb (stylopod, zeugopod, and autopod)²². These regions further separate into the cell clusters that will become the individual muscles of each joint region²². The formation of these muscle cell clusters requires rapid replication, and cell are often produced in surplus, requiring regulation through PCD²². These large areas of muscle mass are termed muscle bellies, which is the bulge in the middle of the muscle between the regions that attach to tendons²².

In addition, muscles require attachment to tendons to inhibit loss of muscle mass and increased apoptosis²². Muscles show an increase in apoptosis when a tendon is severed through surgery²². This suggests that tendon lengthening during treatment of clubfoot may contribute to muscle wasting and atrophy of the calf muscles. The connective tissues of the tendons develop first, regulated by β -catenin and *WNT* signaling, and provide a scaffold where the muscle cells migrate²².

The apoptosis that regulates muscle cell growth is dependent on RA signaling, and inhibition of RA causes a disorganization of the tendon tissues²². The retinoic acid receptor

(*RARβ*) and *CYP26A1* are expressed in the tissues that undergo apoptosis and expression is inversely proportional to the amount of retinoic acid in the tissues²².

Causes of Clubfoot

Clubfoot is a common birth defect although the birth prevalence varies from a low of 1/2500 in African Americans to a high of 1/150 in Polynesians, with a worldwide average rate of approximately 1/1000³⁸⁻⁴¹. Half of all cases have unilateral involvement, and of those the right side is affected more frequently than the left³⁸. Males are affected twice as often as females⁴². Segregation analyses suggest that clubfoot is likely caused by a single gene with reduced penetrance or several other genes with minor effects and environmental influences⁴²⁻⁴⁶. Because males are affected more frequently than males, it is hypothesized that clubfoot follows the Carter (multifactorial) effect, in which females require more susceptibility loci than males⁴⁷. This is supported by family studies that show male children of affected mothers have the highest affection rates, and females of affected fathers have the lowest affection rates⁴⁷. In addition to genetic contribution to the clubfoot model, several environmental factors have been suggested to play a role in clubfoot development⁴⁸⁻⁵¹.

Intrauterine constraint has been suggested as an environmental etiologic factor, but no conclusive data supports this theory.⁵² Seasonal variation has also been suggested to contribute to clubfoot but this has not been supported⁵³⁻⁵⁶. Maternal smoking is associated with clubfoot and increases the odds of having a child with clubfoot (OR 1.3-2.2)⁵⁷⁻⁵⁹. In addition, a family history of clubfoot in a woman who smokes while pregnant increases her risk of having a child with clubfoot by twenty fold⁵⁸. Maternal smoking is not only strongly associated with clubfoot, but it is the only environmental factor shown to contribute to clubfoot with results that are readily reproducible⁵²⁻⁵⁹.

Evidence supporting a genetic etiology underlying clubfoot comes from (1) aggregation of clubfoot in families, (2) twin studies and (3) segregation analyses. Clubfoot can be found throughout multiple generations in families, which suggests that clubfoot is in part genetic⁶⁰. A Danish twin study analyzed 52 twin pairs and found that 17% of monozygotic twins were concordant compared to only 5% of dizygotic twins, suggesting genetics plays a role in clubfoot⁶¹. A second twin study found that monozygotic twins had a higher concordance of 32.5% than that of dizygotic twins (2.9%)^{62,63}. The twin studies vary in percentages due to differences in populations studied, but that is to be expected due to differences in clubfoot etiology between ethnicities and populations. These twin studies suggest genetic liability for clubfoot because monozygotic twins share more genes than dizygotic twins.

Several segregation analyses have been performed with many different conclusions for the populations studied including, a dominant gene, a recessive gene, incomplete penetrance, X-linked, and polygenic inheritance^{43,44,63-65}. One segregation analysis concluded that clubfoot is caused by a major gene that is affected by modifier genes and environmental factors for Caucasians⁴⁴. A study by Palmer supports this finding⁶⁶. However, another study that assessed Hawaiians, Caucasians and Asians found a major gene affect with multifactorial contribution for Hawaiians and Caucasians while no major gene was involved in the Asian etiology⁴³. The segregation analysis on nonHispanic whites and US born Hispanics found similar rates of occurrence under a recessive model with reduced penetrance⁶⁷. Altogether these segregation analyses demonstrate different modes of inheritance for different populations, and most likely there will be an interaction of several genes and environmental factors. These studies clearly suggest a genetic etiology, and

genetic studies are needed to understand the development of clubfoot and improve genetic counseling and risk determination.

Genetic Mapping

Genetic mapping identifies loci that are either linked or associated with a disease or disease susceptibility, and there are several different methods for genetic mapping⁶⁸. Linkage mapping uses the number of meiotic crossover events that take place to identify chromosome regions that harbor susceptibility genes, measured in linkage disequilibrium (LD)⁶⁸. Linkage disequilibrium occurs when two loci are passed together during meiosis more often than by chance due to lack of meiotic crossover between the two loci⁶⁸. Because these events are traced through meiotic events, linkage studies can only be performed in families and large pedigrees with many affected individuals⁶⁸. The best markers to identify linkage are microsatellite polymorphisms, which are short repeats that have varying numbers of repeats in different alleles⁶⁸. These markers are highly polymorphic and provide the most information for linkage analyses⁶⁸. In addition, multipoint analysis can be used to extrapolate information between markers.

Association studies are closely related to linkage studies and are based on populations being derived from founders, and limited meiotic crossovers produce inherited haplotypes over many generations⁶⁸. Association studies work well with case control studies, however association tests can also be performed in samples composed of families and trios⁶⁸. Association studies typically use single nucleotide polymorphisms (SNPs) as markers because they are bi-allelic and are available in high-throughput formats. SNPs are much

more common than microsatellite markers and therefore can be used for fine mapping because several markers within a gene can be selected⁶⁸.

Genome-wide genotyping methods are expensive, and so several methods can be used to retain power of the study and reduce the cost⁶⁸. Tagged SNPs represent a particular haplotype, and genotype information for the other SNPs in that haplotype can be inferred from the genotype of the tagged SNP⁶⁸. In addition, regions of the genome can be targeted through analysis of candidate genes. These may be genes that are in linked regions that need to be fine mapped, genes involved in processes that may lead to the disease if perturbed, or genes that cause a syndrome that has the disease phenotype. These mapping techniques can be used to identify variation that contributes to the disease process.

Previous Genetic Studies

One of the first studies to narrow the search for genetic variation leading to clubfoot was performed by Brewer et al⁶⁹. This study included children who had a variety of syndromes caused by large chromosomal deletion regions. The children were then grouped by phenotypic features, such as clubfoot and then the chromosomal deletion regions were compared⁶⁹. Even though the affected individuals have different syndromes, the overlapping deletion regions between individuals with a shared trait might contain genes that contribute to the idiopathic condition⁶⁹. The study found six large chromosomal deletion regions shared among individuals with syndromic clubfoot: 2q31-33, 3q23-24, 4p16-14, 7p22, 13q33-34 and 18q22-23⁶⁹. Brewer performed a follow up to this study by performing a similar analysis of children with large duplication regions, which found two more regions associated with clubfoot: 6q21-27, 10p15-11⁷⁰.

There have been few genetic mapping studies of idiopathic clubfoot. Deitz et al. performed a segregation analysis on a clubfoot family with four generations available for genotyping, with 13 affected individuals and 41 unaffected individuals⁷¹. This study found two chromosomal regions of interest with LOD scores above 2.0. The authors identified two possible candidate genes, *WNT7A* and *LMX-1*, although further study was needed to assess variations in these genes⁷¹. Shyy et al. followed up this study with the sequencing of two genes in these candidate regions, *WNT7A* and *CAND2*⁷². The sequencing of exons and promoters identified a variant in each gene, although the variants occurred in equal numbers of cases and controls⁷². The authors concluded that these two genes did not contribute to idiopathic clubfoot.

Because smoking has been strongly associated with clubfoot, genes involved in toxin metabolism, if perturbed, could increase susceptibility to clubfoot. N-acetyltransferase (*NAT2*) acetylates toxins including free radicals found in cigarette smoke, and slow acetylation of these molecules can lead to the formation of adducts, which can impair normal development^{73,74}. Specific SNPs within *NAT2* have been shown to lead to slower acetylation of target molecules. Five variants in *NAT2* were genotyped in our clubfoot population, and one of these sites had fewer than expected normal homozygotes in the affected individuals⁷⁵. Additionally, in a small case-control study, there were more slow acetylators than expected in the cases, suggesting that slow acetylators may have a higher risk for clubfoot⁷⁵.

Distal arthrogryposis is a contracture syndrome that includes clubfoot in the phenotype as well as contractures of the hand, which has been shown to be caused by mutations in several different genes involved in the muscle contracture complex⁷⁶. Gurnett et al. conducted a small study, interrogating *MYH3*, *TNNT3*, and *TPM2* (muscle contraction genes) for

association with clubfoot⁷⁶. All exons and 5' and 3' regions were sequenced in 20 idiopathic clubfoot patients⁷⁶. Rare variants were found, but did not segregate with the disease and therefore are not thought to contribute to clubfoot⁷⁶.

A linkage analysis was performed on a five generation family stated to have idiopathic clubfoot, although the proband had bilateral polydactyly and missing the tibia of his right leg and other individuals had bilateral hypoplastic patella⁷⁷. This implies that the family does not have idiopathic clubfoot. The linkage analysis yielded a LOD score of 3.31 on chromosome 5, and *PITX1* was a candidate gene found in the linkage region⁷⁷. *PITX1* is a transcription factor that is required for hindlimb expression, and loss of its expression causes mouse hindlimbs to resemble forelimbs⁷⁷. *PITX1* also has higher expression in the right side of the body, and contributes to differences in development between the two sides such as the ventricles of the heart⁷⁷. A mutation in *PITX1* was found in five affected family members but not in 500 normal controls. The researchers concluded that the findings suggest *PITX1* or the pathway it is involved in plays a role in idiopathic clubfoot, although the family did not have idiopathic clubfoot⁷⁷. They further concluded that *PITX1* might contribute to the right foot being affected more often in unilateral clubfoot due to the higher expression levels in the right limb⁷⁷.

The Hecht lab began systematically analyzing the regions identified by Brewer et al. and interrogating them for genes associated with isolated clubfoot^{69,78}. Beginning with the 2q31-33 region, nine short tandem repeat (STR) markers were selected spanning the region and tested for association in our clubfoot population consisting of nonHispanic white and Hispanic families with or without history of clubfoot⁷⁸. Two STRs segregated with affected individuals, and three genes (Caspase 8, Caspase 10, and *CFLAR*) were located near one of

the STRs. SNPs were selected in these genes and tested for association in the same clubfoot population because these genes are involved in the apoptotic pathway and participate in limb development⁷⁸. One SNP from Caspase (*CASP*) *10* had an altered transmission in the clubfoot population ($p=0.002$)⁷⁸.

Several labs have used retinoic acid, a known teratogen, to induce syndromic clubfoot-like deformities in mice and rats⁷⁹⁻⁸¹. Only one study evaluated protein levels in the exposed offspring who had facial clefts and neural tube defects⁷⁹. Several proteins were down regulated including X-linked inhibitor of apoptosis protein (*XIAP*), troponin T1 (*TNNT1*) and collagen type 2 (*COL2a1*)⁷⁹. Increased apoptosis was also observed in these rats⁷⁹. This study suggests that these genes might be involved in clubfoot, but have not yet been evaluated.

Significance

Clubfoot is a common birth defect that affects about 130,000 newborns throughout the world every year. Treatment modalities such as casting and surgery have improved the long term outcome but residual foot and leg abnormalities often persist^{42,82,83}. Little is known the etiology of clubfoot. Genes are known to play a role in clubfoot, but the challenge is to identify these. If the genetic pathways contributing to clubfoot can be determined, then preconceptual treatments may be developed, or a new method of diagnosis may be developed. *The goal of this project is to define genes that contribute to clubfoot.* In addition, the identification of high risk haplotypes could improve genetic counseling. **This work is significant because it aims to determine the underlying mechanism causing a common birth defect that involves abnormal limb development.**

Brewer et al. identified six large deletion regions that overlapped in individuals with syndromic clubfoot and hypothesized that these regions may contain genes that contribute to

idiopathic clubfoot⁶⁹. Each of the six regions contained genes which are involved in the apoptotic pathway as well as genes that interact with the apoptotic pathway (www.stanford.source.edu). To determine whether variation in these genes plays a role in clubfoot, the SNPs within these apoptotic genes were genotyped and tested for association or linkage to clubfoot. These results are discussed in Chapters 3 and 4.

The 2q31-33 syndromic deletion region from the Brewer study contains the *HOXD* gene cluster which directs axial and limb patterning during development (www.genome.ucsc.edu)^{31,33}. *HOXA* is located on chromosome 7p15-14 and has a redundant function with *HOXD*³³. Mutations in *HOXA* and *HOXD* genes have been associated with six syndromes that involve limb abnormalities, which include synpolydactyly and brachydactyly (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim>). The association of *HOX* mutations with limb anomalies led us to interrogate the *HOXA* and *HOXD* gene clusters in our clubfoot samples. The results of these studies are found in Chapter 5.

Chapter 2: Materials and Methods

Study Population

Probands with clubfoot were identified through Shriners Hospitals for Children of Houston and Los Angeles and the Scottish Rite Hospital of Dallas and are designated as the “discovery population”. Affected individuals were diagnosed with clubfoot through clinical and radiographic diagnosis. Individuals with chromosomal abnormalities, syndromes or postnatal events associated with clubfoot were excluded. Ethnicity was self-reported and informed consent was obtained. Two generation pedigrees were obtained for all probands, and these pedigrees were classified into those with affected relatives (multiplex family history) or those without affected relatives (simplex trios). Pedigrees were extended for multiplex families to include all individuals affected.

A validation clubfoot population of 144 nonHispanic white trios was obtained from Washington University to test positive results found in the discovery population. For the *HoxD10* M319K mutation analysis, two positive controls with congenital vertical talus (CVT) with this mutation were obtained from Dr. Dobbs at Washington University. 595 unrelated, unaffected negative controls were ascertained through the cleft lip and palate clinics at Children's Hospital, Boston, Texas Children's Hospital, Houston, and the University of Texas Craniofacial Clinic, Houston.

Samples

DNA was extracted from blood or saliva samples collected from participating individuals. DNA was extracted from blood using the DNA Isolation Kit for Mammalian Blood (Roche, Palo Alto, CA) or saliva with Oragene Purifier (DNA Genotek Inc., Ottawa, Ontario, Canada). Each of these kits was used according to the manufacturer’s instructions.

DNA samples were quantified using the Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and checked for degradation by running on a 2% agarose gel. DNA was then stored at -20°C.

Gene Selection

Brewer et al. studied children with large deletion regions clubfoot to try and narrow down genomic regions associated with idiopathic clubfoot⁶⁹. They aligned chromosomal deletion regions in these children and identified the smallest overlapping regions⁶⁹. These regions might contain genes that contribute to idiopathic clubfoot. Candidate genes within these regions, including HOX and apoptotic genes, were interrogated for association with clubfoot. These studies are described in Chapters 3, 4 and 5.

Genotyping

Genotyping was performed using single nucleotide polymorphisms (SNPs) as genetic markers. SNPs were selected using the National Center for BioInformatics (NCBI, <http://www.ncbi.nlm.nih.gov>) and Ensembl (<http://www.ensembl.org>) websites. SNPs selected were based on heterozygosity (>0.3), placement in gene, tagging ability and coverage of gene. Software programs Haploview⁸⁴ and SNPBrowser were also used for SNP selection. Genotyping was performed using TaqMan® SNP Genotyping Assays and SNPlex™ Genotyping System (Applied Biosystems, Foster City, CA). TaqMan® results were analyzed on the 7900HT using SDS 2.1 (Applied Biosystems, Foster City, CA). SNPlex™ results were analyzed on a 3730 using GeneMapper® 4.0 (Applied Biosystems, Foster City, CA). Both TaqMan® and SNPlex™ reactions were performed according to standard protocols. The *HoxD10* M319K mutation was detected using a custom TaqMan®

assay. The primers were designed using File Builder 3.0 (Applied Biosystems, Foster City, CA).

Statistical Analyses

The data was originally analyzed as a whole and then probands were stratified by ethnicity alone and ethnicity and family history. Allele frequencies and Hardy-Weinberg Equilibrium (HWE) were calculated using SAS (v9.1). Pairwise linkage disequilibrium values (D' and r^2) were calculated using GOLD⁸⁵.

Different methods for assessing linkage and/or association were performed in order to obtain the greatest amount of information from this dataset. Parametric and non-parametric linkage analyses were conducted using Merlin⁸⁶. Graphical Overview of Linkage Disequilibrium (GOLD) was used to calculate the disequilibrium coefficient (D') between pairs of SNPs⁸⁷. The pedigree disequilibrium test (PDT) and the family based association test (FBAT) were used to determine association in clubfoot families^{88,89}. The PDT tests for altered transmission of alleles within pedigrees. The Geno-PDT is a function within the PDT, and tests specific genotypes is more accurate when looking at dominant and recessive models⁷⁵. Family based associated test (FBAT) was used to test for association and the HBAT function within FBAT was used to test haplotypes⁹⁰. HBAT and FBAT were run under a recessive model.

Association in the Presence of Linkage (APL) was used because the program incorporates data even when a parental genotype is missing⁹¹. Altered transmission of pairwise haplotypes within a gene was analyzed using APL. Positive associated SNPs ($p < 0.05$) were then analyzed for gene-gene interaction using Generalized Estimating Equations (GEE)⁹²

Sequencing

The region surrounding mir196-b was amplified using polymerase chain reaction (PCR) with primers: Fwd- 5'-GCTCGCTGGGCTGCAAGATTTGG-3' and Rvs- 5'-CTGCGGAGAAAGACACGAGGCTC-3'. The PCR was performed using the following program: 94°C for 2:00 [98°C for 0:10, 55°C ± 1°C for 0:30, 72°C for 1:00] for 35 cycles and 72°C for 10:00. Five percent DMSO was added to the reaction because of the high G-C content of the DNA. The 552bp product was sequenced by Lone Star Labs, Inc. (Houston, TX). Sequence was analyzed using Sequencher (Gene Codes, Ann Arbor, Michigan).

Transcription Factor Binding Prediction

Both the (-2) miR196-b and SNP16 variants are in potential promoter regions and could affect putative binding sites. Three *in silico* prediction programs, Alibaba2, Patch and Transcription Element Search Software (TESS), were used to assess whether ancestral or alternate sequences changed or generated a transcription factor binding site⁹³⁻⁹⁵. The ancestral allele is defined by NCBI as the allele that is found in chimpanzee and is thought to be shared by the common ancestor (http://www.ncbi.nlm.nih.gov.ezproxyhost.library.tmc.edu/bookshelf/br.fcgi?book=helpsnpfag&part=Content#Content.Ancstral_Allele_Dat). The *in silico* testing provides theoretical data which needs to be confirmed with functional testing.

Chapter 3: Mitochondrial Mediated Apoptotic Pathway and Clubfoot

This chapter is a presentation of work from a previously published paper. For more information please refer to: Ester, AR, et al. Apoptotic gene analysis in idiopathic talipes equinovarus (clubfoot). Current Orthopedic Related Research. 2007 Sep; 462: 32-7.

Background of Apoptosis and clubfoot

Deletion Regions Associated with Clubfoot

Cytogenetic deletion mapping has been used in mapping many diseases, although a comprehensive study of these deletions had not been performed in birth defects. Therefore, Brewer et al. decided to study children with different birth defects caused by chromosome deletions⁶⁹. The study consisted of 1,753 children with 47 different phenotypes, although some children had multiple malformations⁶⁹. Similar malformations were grouped together and assessed for common deletion regions⁶⁹. For clubfoot, six chromosomal deletion regions were found: 2q31-33, 3q23-24, 4p16-14, 7p22, 13q33-34, and 18q22-23, and are candidate regions for harboring genes involved in isolated clubfoot⁶⁹. However, there are 294 known genes in these regions, so further mapping is required to identify the genes associated with clubfoot.

Fine Mapping of Chromosome 2 Deletion Region

Interrogation of the chromosome 2 deletion region associated with clubfoot was performed to narrow the clubfoot candidate region⁷⁸. Nine microsatellite markers spanning the 2q31-33 region were genotyped in 83 trios and 57 families with clubfoot. GATA149B10 and D2S1371 were significantly associated with clubfoot⁷⁸. However, these markers only narrowed the region to 6cM, and further fine mapping was needed. Three candidate genes were identified near one of the satellite markers: *Casp8*, *Casp10*, and *CFLAR*, which are all involved in apoptosis⁷⁸. Eleven SNPs across these three genes were analyzed, and rs3769825 in *Casp8* and rs3900115 and rs3731714 in *Casp10* were associated with clubfoot⁷⁸. These results suggest a role of apoptosis in clubfoot.

Apoptosis

Apoptosis or programmed cell death (PCD) is the process by which cells die in response to cell signals⁹⁶. This process includes chromosome condensation, cell shrinkage, membrane blebbing, formation of apoptotic bodies and degradation of these bodies by surrounding cells⁹⁶. These apoptotic bodies are then taken up and digested by neighboring phagocytes⁹⁷. This process is different from necrosis which does not follow this defined progression of cell death⁹⁸. Apoptosis regulates cell proliferation during development and plays an active role in the homeostasis of cells after embryogenesis. Loss of apoptosis can lead to cancer⁹⁸.

Mitochondrial Mediated Apoptosis

There are six protein motifs that are associated with apoptosis: caspase (CASP), caspase recruitment domain, death domain (DD), death effector domain (DED), BIR domain and BCL-2 homology domain⁹⁸. DDs are required for coupling of death receptors and inter-protein binding of the death-signaling complex, and without them the apoptotic signal could not be transmitted⁹⁹. The extrinsic mitochondrial mediated apoptotic pathway begins with the coupling and self-activation of death receptors in response to their ligands which are released in response to cellular signals⁹⁸. The activated receptors then recruit death domain containing proteins, which activate the extrinsic mitochondria mediated apoptotic pathway (Fig. 3.1)⁹⁸.

Activator Caspases

Caspases (*CASP*) 2, 8, 9, and 10 are known as activator caspases and respond to external

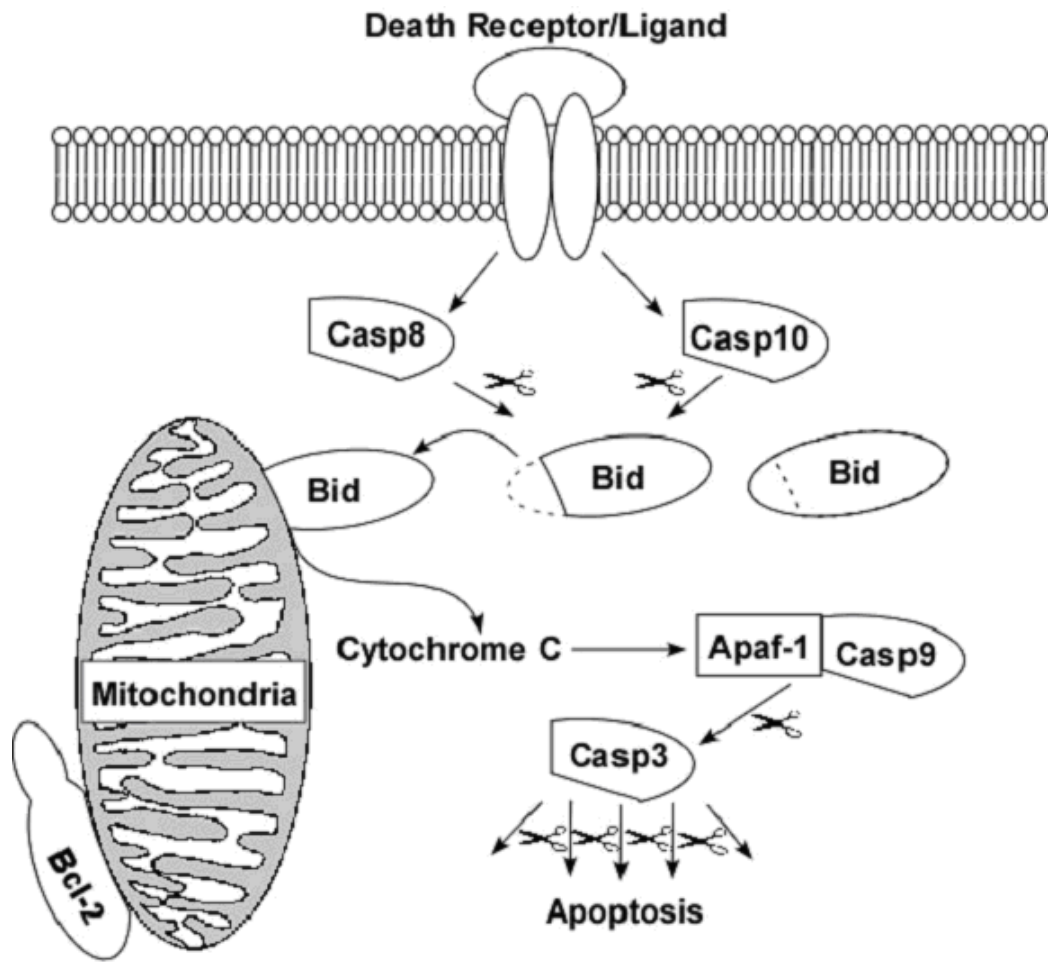


Fig. 3.1. Mitochondrial mediated apoptotic pathway

signals to initiate apoptosis⁹⁷. Caspases cleave after aspartic acid residues and target RNA splicing, DNA repair proteins and other caspases¹⁰⁰. Caspases are synthesized as inert proteins, procaspases, and activation of caspases lead to the cellular changes seen during PCD^{97,100}. Activator caspases have long prodomains that recruit adaptor proteins, and Casp8 and 10 contain death effector domains (DEDs), and the adaptor domains are responsible for activating the caspases⁹⁷. The activated death receptor binds CASP8 and 10 through the DED, activating the proteins and cleaving BID (BH3-interacting domain death agonist), a BCL2 family protein⁹⁷.

BID

BID participates in a mitotic checkpoint and maintains genomic stability, but its main role is in the extrinsic mitochondrial mediated apoptotic pathway¹⁰¹. Full length BID has been shown to have minor apoptotic activity, but once BID is cleaved by CASP8, the transmembrane domain is able to incorporate into the outer mitochondrial membrane¹⁰¹. This insertion stimulates a cascade, leading to permeabilization of the mitochondrial membrane¹⁰¹. Once the membrane becomes permeable, cytochrome c is released from the mitochondria¹⁰¹. This allows the apoptotic cascade to continue, leading to the eventual degradation of the cell¹⁰¹.

BCL-2

BCL-2 is an anti-apoptotic member of the BCL-2 family, and is localized to surfaces of membranes, including the mitochondria, nucleus and endoplasmic reticulum¹⁰². BCL-2 functions to keep the mitochondrial membrane intact, thus preventing the release of cytochrome c during normal cell functions¹⁰³. BCL-2 is targeted by activated BID, and once

BID enters the mitochondria membrane, Bcl-2 can no longer contain cytochrome c within the mitochondria¹⁰³. Once cytochrome c is released, CASP9 and APAF-1 are activated¹⁰³.

CASP9 and APAF-1

CASP9 is the most studied and best characterized of the caspases, and activation requires incorporation into the apoptosome¹⁰⁴. The apoptosome consists of CASP9, cytochrome c, APAF-1, and cofactor dATP/ATP¹⁰⁴. APAF-1 binds to procaspase 9 in the presence of cytochrome c. The apoptosome can only form when the mitochondrial mediated apoptotic pathway has been activated and cytochrome c has been released from the mitochondria¹⁰⁴. Once a homodimer of procaspase 9 assembles into the apoptosome, the procaspase has a low level basal activity that is three times higher than that of unbound procaspase 9¹⁰⁴. This allows auto-activation of CASP9, creating an active apoptosome¹⁰⁴. The apoptosome then activates CASP3¹⁰⁴.

CASP3

CASP3 has a key role in apoptosis, and is known as an effector caspase¹⁰⁵. CASP3 cleaves many substrates including poly ADP ribose polymerase (PARP), which is cleaved during apoptosis¹⁰⁵. Once these substrates are cleaved, the cell undergoes many changes including membrane blebbing⁹⁶. The cell breaks into many different apoptotic bodies, which are then degraded by phagocytes⁹⁶.

Study Design

To assess mitochondrial mediated apoptotic genes for association with clubfoot, SNPs were selected spanning seven apoptotic genes, *CASP8*, *CASP10*, *BID*, *BCL-2*, *APAF-1*, *CASP9* and *CASP3*. Because *CASP8* and *CASP10* were already tested in a smaller sample

set, only the significant SNPs were rerun in the expanded sample. The data set consisted of 82 nonHispanic white (NHW) simplex trios, 88 NHW multiplex families, 128 Hispanic simplex trios and 51 Hispanic multiplex families. Forty SNPs in seven genes were genotyped in these families (Table 3.1).

Results

Allele frequencies in NHW and Hispanics were compared, and because of the large number of comparisons, a conservative threshold of $p < 0.00125$ was used to assess significance¹⁰⁶. No differences in allele frequencies were detected between NHW and Hispanics for the SNPs in *CASP8*, *9* and *10*¹⁰⁶. However, allele frequencies differed for five out of eight SNPs in *CASP3*, five out of seven in *APAF-1*, two out of five in *BCL-2* and two out of eight in *BID* (data not shown)¹⁰⁶. The two populations were analyzed separately because of these allele differences. Pairwise linkage disequilibrium (LD) was calculated using unaffected individuals, and the patterns of LD were similar between NHW and Hispanics¹⁰⁶. A representative LD plot for *CASP9* is shown in Table 3.2, which shows that the LD patterns are similar between NHW and Hispanics (all LD plots are found in Appendix A).

Parametric linkage analysis, which assumes a specific mode of inheritance, did not detect linkage for any of the SNPs tested¹⁰⁶. Non-parametric linkage analysis found suggestive evidence for association for rs1049253 and rs1049216 ($p=0.07$ and 0.06 , respectively) in *CASP3* in the Hispanic sample (complete data not shown)¹⁰⁶.

Table 3.1. Location of SNPs analyzed, predicted protein changes and differences in allele frequencies between NHW and Hispanics.

Gene/Chr Region	SNP	Allele	BP Position	Location	AA Change	NHW	Hisp.	P-Value
<i>CASP9</i> 1p36.21	rs35718454	T/G	15725876	Upstream	-	0.534	0.575	0.092
	rs1052571	G/A	15723200	Exon 2	A28V	0.584	0.622	0.307
	rs4646008	G/A	15717314	Exon 2	S99L	0.996	0.999	0.254
	rs2308941	G/A	15717305	Exon 2	T102I	0.977	0.981	0.651
	rs2020897	C/G	15717268	Exon 2	E114D	0.993	0.994	0.475
	rs1820204	A/G	15717202	Exon 2	F136L	0.522	0.587	0.047
	rs2042370	G/A	15714329	Intron 2	-	0.564	0.612	0.135
	rs2308950	C/T	15706093	Exon 4	R173H	0.970	0.988	0.161
	rs4233533	G/A	15701774	Intron 6	-	0.711	0.738	0.723
<i>CASP10</i> 2q33.1	rs3900115	A/G	201758922	Exon 3	Synon.	0.541	0.612	0.051
	rs3731714	A/G	201769065	Intron 5	-	0.772	0.769	0.916
<i>CASP8</i> 2q33.1	rs3769825	C/T	201819625	Intron 9	-	0.503	0.538	0.346
<i>CASP3</i> 4q35.1	rs1049253	C/T	185785945	3' UTR	-	0.796	0.851	0.092
	rs1049216	T/C	185787083	3' UTR	-	0.716	0.439	< 0.00001
	rs1049210	C/A	185789219	Exon 7	E190D	0.995	0.998	0.820
	rs1405944	T/A	185790347	Intron 5	-	0.512	0.249	< 0.00001
	rs2696057	G/C	185792828	Intron 4	-	0.834	0.809	0.130
	rs2720378	C/G	185805107	Intron 2	-	0.714	0.434	< 0.00001
	rs4647602	A/C	185806795	Intron 1	-	0.921	0.651	< 0.00001
	rs1405937	G/C	185808932	Upstream	-	0.837	0.562	< 0.00001
<i>APAF-1</i> 12q23.1	rs7310804	A/G	97543189	3' UTR	-	0.514	0.458	0.108
	rs2278361	A/G	97567338	Intron 2	-	0.789	0.712	< 0.00001
	rs2288729	T/C	97591721	Intron 10	-	0.652	0.618	0.00008
	rs6538879	A/G	97612225	Intron 14	-	0.750	0.732	0.00072
	rs3782558	G/C	97629924	Intron 17	-	0.541	0.561	0.061
	rs1866477	G/T	97643964	Intron 22	-	0.885	0.929	0.00076
	rs7968661	A/G	97661515	Downstream	-	0.680	0.628	0.00003
	<i>BCL-2</i> 18q21.33	rs1564483	A/G	58945634	3' UTR	-	0.742	0.884
rs8083946		G/A	59056901	Intron 1	-	0.606	0.548	0.936
rs1801018		T/C	59136859	Exon 3	Synon.	0.579	0.575	0.274
rs2551402		C/A	59141002	Downstream	-	0.515	0.631	0.00004
rs1809319		C/T	59173614	Downstream	-	0.663	0.600	0.005
<i>BID</i> 22q11.21	rs8919	G/A	16593057	Upstream	-	0.532	0.432	0.007
	rs181399	T/C	16605318	Intron 3	-	0.821	0.858	0.941
	rs2072392	A/G	16606612	Exon 3	Synon.	0.973	0.963	0.509
	rs8190315	T/C	16606764	Exon 3	S56G	0.976	0.982	0.322
	rs181405	G/A	16613000	Intron 1	-	0.535	0.439	0.075
	rs181410	A/T	16617436	Intron 1	-	0.655	0.804	0.007
	rs5747351	A/G	16626375	Intron 1	-	0.601	0.436	< 0.00001
	rs3788284	G/C	16632103	Intron 1	-	0.555	0.433	0.00023

AA = amino acid, Synon.=synonymous, NHW= nonHispanic white, Hisp.=Hispanic; Chromosome position noted under gene

All single SNPs with a $p < 0.1$ are shown in Table 3.3. rs3769825 (*CASP3*) and rs2551402 (*BCL-2*) gave p -values of 0.05 and 0.07, respectively in the Hispanic simplex cases (Table 3.3)¹⁰⁶. No SNPs had an altered transmission in the Hispanic multiplex families. In the nonHispanic white simplex trios, rs2278361 and rs2288729 in *APAF-1* and rs2551402 in *BCL-2* yielded p -values of 0.03, 0.06 and 0.06, respectively¹⁰⁶. SNPs rs2278361 and rs2288729 in *APAF-1* were in strong LD (Appendix A). In the nonHispanic white multiplex families, rs4233533 in *CASP9* and rs2696057 in *CASP3* gave nonsignificant p -values of 0.09 and 0.08, respectively¹⁰⁶. Results from the PDT analysis were similar to the FBAT results (Table 3.3)¹⁰⁶. However, two SNPs with suggestive p -values were identified in the Hispanic multiplex families; one in *CASP10* (rs3731714) and the other in *BID* (rs2072392), which were not detected by FBAT¹⁰⁶.

Haplotypes were generated using HBAT and are shown in Table 3.4. The haplotypes are not true haplotypes because the SNPs do not reside in same gene or on the same chromosome. However, in complex diseases, the underlying etiology is assumed to involve variants in multiple genes which are not necessarily on the same chromosome. By identifying these haplotypes, we can begin to determine gene-gene interactions¹⁰⁶. SNPs with $p \leq 0.1$ in one population were evaluated in all populations. In the Hispanic simplex trios, an overall altered transmission of both haplotypes of rs3739825 and rs2551402 in the *CASP3* and *BCL-2* haplotype was found ($p=0.017$)¹⁰⁶. In the NHW simplex trios, altered transmission of haplotypes for rs2278361 (*APAF-1*) and rs2551402 (*BCL-2*) and rs2288729 (*APAF-1*) and rs2551402 (*BCL-2*) were found ($p=0.03$, $p=0.02$ respectively)¹⁰⁶. Because rs2278361 and rs2288729 are in strong LD, only one was considered in each of the

Table 3.2. LD plot for *CASP9*.

	hcv2845956	rs4233533	rs2308950	rs2042370	rs1820204	rs2020897	rs2308941	rs4646008	rs1052571
hcv2845956		0.96	1.00	0.98	0.95	0.95	1.00	0.85	0.98
rs4233533	0.92		1.00	0.82	1.00	1.00	1.00	1.00	0.82
rs2308950	0.18	0.10		0.92	1.00	0.48	1.00	1.00	1.00
rs2042370	0.97	0.91	1.00		1.00	1.00	1.00	1.00	0.93
rs1820204	0.97	0.98	0.22	0.99		0.00	1.00	1.00	0.98
rs2020897	0.99	1.00	1.00	1.00	1.00		1.00	1.00	1.00
rs2308941	1.00	0.31	0.23	0.29	1.00	1.00		1.00	1.00
rs4646008	0.00	0.00	0.00	0.00	0.00	0.00	0.00		1.00
rs1052571	0.93	0.87	1.00	0.95	0.96	1.00	0.36	0.00	

NonHispanic whites shown above the diagonal, Hispanics shown below the diagonal.
 $p < 0.05$ shown in yellow.

Table 3.3. Apoptotic single SNP results by population.

Group	SNP	FBAT	PDT	
			Global	Summed
Hispanic-Simplex	rs3769825 (<i>CASP8</i>)	0.05	0.09	0.03
	rs2551402 (<i>BCL-2</i>)	0.07	0.08	0.06
Hispanic-FH	rs3731714 (<i>CASP10</i>)	-	0.06	> 0.1
	rs2072392 (<i>BID</i>)	-	0.07	0.05
NHW-Simplex	rs2278361* (<i>APAF-1</i>)	0.03	0.04	0.06
	rs2288729* (<i>APAF-1</i>)	0.06	0.08	0.1
	rs2551402 (<i>BCL-2</i>)	0.06	0.07	0.04
NHW-FH	rs4233533 (<i>CASP9</i>)	0.09	> 0.1	> 0.1
	rs2696057 (<i>CASP3</i>)	0.08	0.08	0.05

*SNPs in LD, NHW=nonHispanic white

Table 3.4. Apoptotic haplotype analysis.

Group	SNPs	Haplotype	p Value	Transmitted		Overall p-Value
				Obs.	Exp.	
Hispanic-Trios	rs3739825/rs2551402	1-2	0.0019	42	52	0.017
	<i>CASP8/BCL-2</i>	2-1	0.059	30	25	
NHW-Trios	rs2278361/rs2551402	1-2	0.03	33	36	ns
	<i>APAF-1/BCL-2</i>					
NHW-FHx	rs2288729/rs2551402	1-1	0.02	17	12	ns
	<i>APAF-1/BCL-2</i>					
NHW-FHx	rs4233533/rs2696057	2-2	0.02	83	74	ns
	<i>CASP9/CASP3</i>					

Obs.=observed, Exp.=expected, NHW= nonHispanic white, FHx=multiplex families, ns=not significant

haplotypes. Lastly, in the NHW multiplex families, altered transmission of a *CASP9* and *CASP3* haplotype (rs4233533/rs2696057) was detected ($p=0.02$)¹⁰⁶.

EMDR analysis was utilized to generate 1-, 2-, 3- and 4-locus models that discriminate between affecteds and unaffecteds for simplex trios. This method identifies which SNPs depending on the number of loci considered are best at segregating the affected from the nonaffected individuals¹⁰⁶. This analysis can identify high risk haplotypes by categorizing which SNPs are most likely to confer risk. As shown in Table 3.5, none of the SNPs represented under different models produced significant results. Based on our results, rs3769825 in *CASP8* was identified as a SNP of interest¹⁰⁶.

Discussion

In this study, we interrogated seven apoptotic genes, *CASP3*, *CASP8*, *CASP9*, *CASP10*, *BID*, *BCL-2* and *APAF-1*, using 40 SNPs and tested for linkage and association with clubfoot¹⁰⁶. Suggestive evidence for association was found for a SNP in each of the seven genes, *CASP3*, *CASP8*, *CASP9*, *CASP10*, *BID*, *BCL-2* and *APAF-1*¹⁰⁶. Gene-gene interactions were identified with altered transmission of multi-gene haplotypes in both NHW and Hispanics¹⁰⁶. Lastly, one SNP, rs3769825 in *CASP8* was also found to be the best discriminator between affecteds and unaffecteds in the Hispanic simplex trios¹⁰⁶. All together these results suggest that the interaction of several genes within the mitochondrial mediated apoptotic pathway influences the development of clubfoot¹⁰⁶.

Of interest, a previous analyses identified altered transmission of rs3900115 in *CASP10* in three of the four sample groups tested in clubfoot families.¹⁰⁷ While only the Hispanic multiplex families gave suggestive results, this study identified a different *CASP10* SNP (rs3731714) as the best single locus model (although this result was not significant)¹⁰⁶. This

Table 3.5. High risk haplotypes*.

Model	NHW			
1 locus	rs3731714 (<i>CASP10</i>)			
2 locus	rs7968661 (<i>APAF-1</i>)	rs1809319 (<i>BCL-2</i>)		
3 locus	rs3782558 (<i>APAF-1</i>)	rs7968661 (<i>APAF-1</i>)	rs1809319 (<i>BCL-2</i>)	
4 locus	rs4233533 (<i>CASP9</i>)	rs1405937 (<i>CASP3</i>)	rs8083946 (<i>BCL-2</i>)	rs181405 (<i>BID</i>)
Model	Hispanic			
1 locus	rs3769825 (<i>CASP8</i>)			
2 locus	rs1052571 (<i>CASP9</i>)	rs3769825 (<i>CASP8</i>)		
3 locus	rs2720378 (<i>CASP3</i>)	rs8919 (<i>BID</i>)	rs5747351 (<i>BID</i>)	
4 locus	rs1049216 (<i>CASP3</i>)	rs2720378 (<i>CASP3</i>)	rs8919 (<i>BID</i>)	rs5747351 (<i>BID</i>)

NHW=nonHispanic white, *EMDR analysis

lack of consistent findings may reflect a difference in the clubfoot data sets that have expanded as more samples were collected. Since other SNPs within genes in this pathway have yielded suggestive results, we are encouraged that this pathway contributes to the clubfoot¹⁰⁶.

In complex diseases, it is expected that perturbation of multiple interacting genes are necessary for the birth defect and may reflect a high risk haplotype¹⁰⁶. The haplotype based function of FBAT (HBAT) can be used to identify altered transmission of haplotypes in both families and trios. “Multifactor dimensionality reduction (MDR) analysis can also detect at-risk haplotypes constructed from multiple genes, but the haplotypes are created by using a case-control based algorithm instead of family based algorithms such as used in HBAT”¹⁰⁶.

The findings of this study and the known role of apoptosis in limb and muscle development strongly suggest that further interrogation studies of additional apoptotic genes and upstream signaling factors that activate apoptosis are necessary and are the focus of studies described in Chapter 4.

*Chapter 4: Analysis of Candidate Genes in Clubfoot
Deletion Regions*

Introduction

Isolated clubfoot is a common birth defect, occurring in 1/700-1000 live births affecting 135,000 newborns each year^{38,40,65,108}. Talipes equinovarus (TEV) is identified by three clinical foot characteristics: forefoot adduction, hindfoot varus and hindfoot equinus¹⁰⁹. The tarsal joint is also misaligned and the arch is often more concave¹¹⁰. Calf muscles are frequently hypoplastic and remain smaller throughout life¹¹⁰. Isolated clubfoot is distinct from syndromic clubfoot in that it occurs without any other anomalies. Males are affected twice as often as females and the frequency varies across ethnicities⁶⁵. Treatment consists of serial castings for six weeks with casts being changed every week¹¹⁰. Soft tissue releases, bony procedures and/or tendon transfers are sometimes needed following serial casting and bracing¹¹⁰.

Clubfoot is a complex birth defect caused by both genetic and environmental factors⁶⁵. Several environmental factors have been suggested to contribute to clubfoot, but only maternal smoking has been consistently associated⁵⁷⁻⁵⁹. Evidence supporting a genetic etiology for clubfoot comes from (1) aggregation of clubfoot in families, (2) twin studies which demonstrate a 32.5% concordance in monozygotic twins compared to 2.9% in dizygotic twins and (3) segregation analyses, which have suggested that clubfoot is most likely caused by a single gene with major effects as well as other genes with minor effects and environmental factors^{44,45,58,67}.

A previous study interrogated minimal overlapping chromosomal deletion regions in individuals with large deletions that had clubfoot as a phenotype. That study found six large chromosomal deletion regions, 2q31-33, 3q23-24, 4p16-14, 7p22, 13q33-34 and 18q22-23, shared among individuals with syndromic clubfoot⁶⁹. Investigation of those regions using Ensembl database (www.ensembl.org) identified 194 known genes⁶⁹. Of these genes, there were 52 candidate genes involved in apoptosis, muscle development, morphogenesis and cell

proliferation (www.source.stanford.edu). Interestingly, apoptotic pathway genes were identified in each of the six deletion regions which was remarkable because apoptotic genes have been previously associated with clubfoot (Table 4.1 and Fig. 4.1) ¹⁰⁶.

The 29 candidate apoptotic genes can be broken down into several groups: immune response

2q31-33	3q23-34	4p14-16	7p22	13q33-34	18q22-23
<i>ZAK</i>	<i>RNF7</i>	<i>TLR10</i>	<i>RBAK</i>	<i>ING1</i>	<i>SOCS6</i>
<i>CERKL</i>	<i>ATR</i>	<i>TLR1</i>	<i>TRIAD3</i>	<i>TFDP1</i>	<i>NFAT2</i>
<i>GULP1</i>	<i>SSB4</i>	<i>TLR6</i>	<i>CARMA1</i>	<i>TNFSF13B</i>	<i>TNFRSF11A</i>
<i>SUMO1</i>	<i>TFDP2</i>	<i>WDR19</i>			
<i>ATF</i>		<i>HIP2</i>			
<i>CREB1</i>		<i>EMP</i>			
<i>STAT1</i>		<i>HTT</i>			
<i>STAT4</i>		<i>TNIP2</i>			

genes that stimulate apoptosis, transcription factors, genes involved in engulfment of apoptotic bodies, and other apoptotic genes. A summary about each of these genes are presented below.

Immunity Related Genes

ZAK

Leucine-zipper sterile α -motif (ZAK) is a serine/threonine kinase, which activates the c-Jun N-terminal kinase (JNK) pathway and the transcription factor NF κ B through the MAP kinase kinase (MKK) pathway¹¹¹. Activation of ZAK can lead to cell arrest in the G2/M stage of the cell cycle, and prolonged expression of ZAK induces apoptosis through the JNK pathway by activating MAPKK 7 (MKK7)¹¹¹.

SUMO1

Small ubiquitin related modifier 1 (SUMO1) can be conjugated to proteins, leading to a regulation of these proteins¹¹². This attachment is processed through several enzymes similar to ubiquitination, although “SUMOylation does not lead to degradation”¹¹². SUMO1 has been shown to interact with DAXX, which is a transcriptional repressor involved in the

apoptotic pathway (www.source.stanford.edu)¹¹³. In addition, SUMO1 sumoylates NFκB, which is a transcription factor that regulates apoptosis and inflammation¹¹⁴.

RNF7

Ring finger protein 7 (RNF7) is also known as sensitive to apoptosis gene (SAG), and is an E3 ubiquitin ligase¹¹⁵. Ubiquitin is a protein that is ligated to other proteins through a series of three ligases and marks the protein for degradation¹¹⁵. RNF7 ubiquitinates c-Jun and inhibits AP-1 and cell proliferation¹¹⁵. In addition, RNF7 conjugates ubiquitin to the inhibitor of NFκB, increasing the levels of NFκB and inhibiting apoptosis¹¹⁵.

TLR Family

The canonical pathway for toll-like receptors (TLRs) involves the anti-microbial immune response, however recent studies have shown that TLRs can activate apoptosis¹¹⁶. Apoptosis is thought to be activated during an immune response to limit the pathologic effects of the immune system¹¹⁶. TLRs activate adapter proteins that bind and activate IRAK kinases, which then activate TRAF6, a ubiquitin ligase¹¹⁶. This leads to the canonical activation of NFκB, which activates MAPKKs and then finally apoptosis¹¹⁶. So far, only TLRs 2, 3, 4, 7, 8 and 9 have been identified as activators of this pathway¹¹⁶. TLR 1, 6 and 10 are in the clubfoot deletion regions, but the role of these TLR family members in apoptosis is not known.

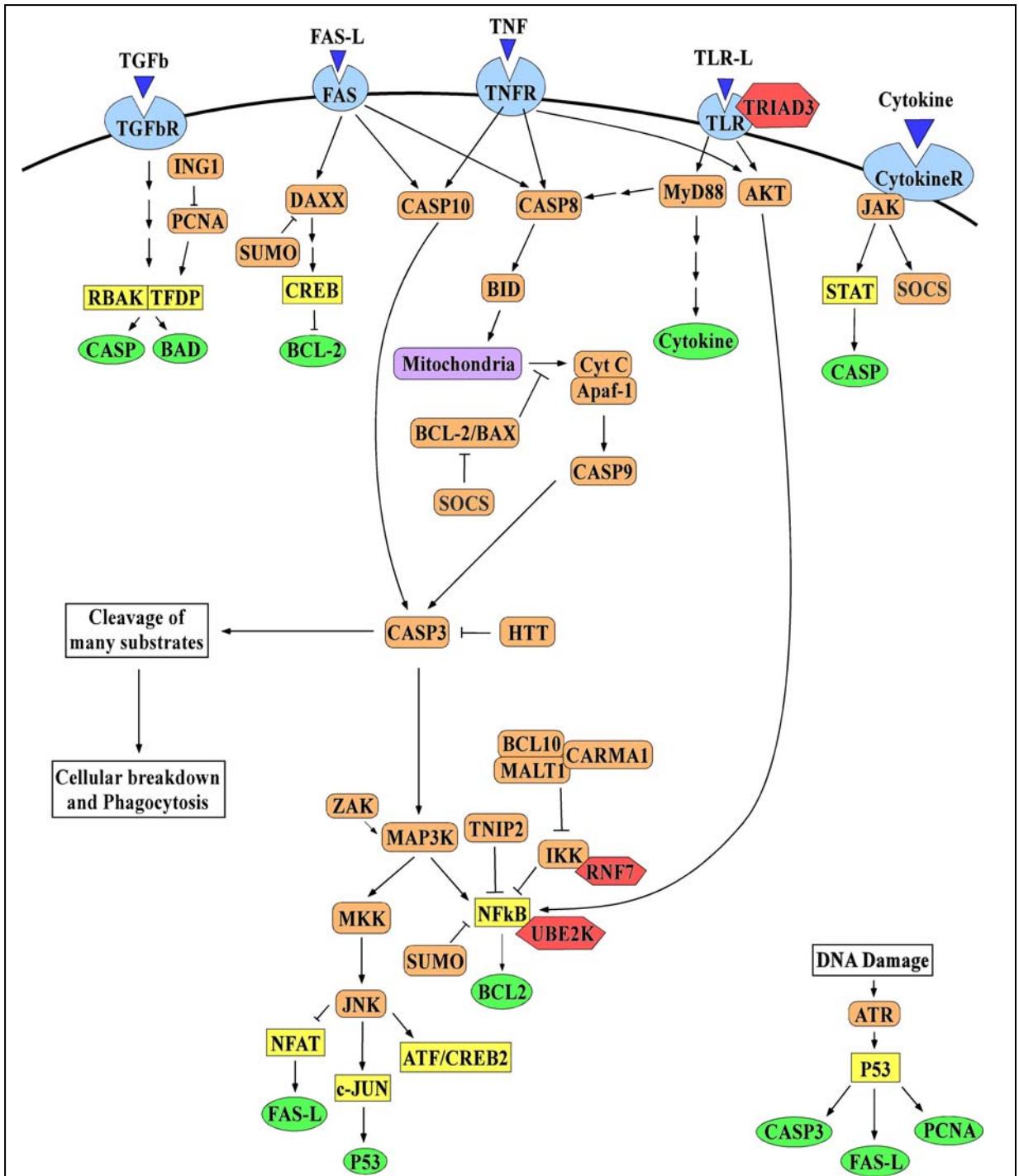


Fig. 4.1. Apoptotic gene pathway in clubfoot chromosomal deletion regions. Interactions between apoptotic, anti-apoptotic and immunity genes found in each of the clubfoot deletion regions. Arch at the top of the page denotes the cell membrane. Color codes for genes are: red = ubiquitin ligase, orange = protein, yellow = transcription factor, green = transcription factor target, blue = receptor, dark blue = ligand

UBE2K

Ubiquitin ligase Kinase 2 (UBE2K) was initially discovered to interact with HTT, but its targets also include the precursor of NF κ B¹¹⁷. Once the ubiquitin is ligated to NF κ B it is rapidly degraded into a smaller, active form¹¹⁷. However, activated NF κ B is sequestered in the cytoplasm by I κ B as a second mechanism of control¹¹⁷. UBE2K is critical for NF κ B activation, and NF κ B acts as an anti-apoptotic factor by increasing expression of BCL-2.

TNIP2

TNIP2 or A-20 binding inhibitor of NF κ B (ABIN2) promotes apoptosis through the inhibition of NF κ B¹¹⁸. TNIP family members are involved in the negative feedback loop of NF κ B that prevents overexpression that leads to immunological disorders¹¹⁸. However, TNIP2 is not activated by NF κ B, although still activates apoptosis through NF κ B inhibition¹¹⁸.

TRIAD3

TRIAD3 (zinc finger protein inhibiting NF κ B) is an E3 ubiquitin protein ligase¹¹⁹. The main role for TRIAD3 is regulating TLRs activity through degradation of TLRs¹¹⁹. In addition, TRIAD3 prevents the cell from undergoing apoptosis by downregulating signaling molecules involved in the TLR activation of NF κ B¹²⁰.

CARMA1

CARMA1 is also known as caspase recruitment domain 11 (CARD11), and complexes with BCL10 and MALT1 (paracaspase)¹²¹. This complex activates IKK, which releases repression of NF κ B and leads to the immune response¹²¹. “The stimulation of surface receptors on immune cells triggers not only changes gene

expression but also morphological changes through the reorganization of the actin cytoskeleton, thereby contributing to efficient cellular activation¹²¹.” This suggests that the genes involved in the immune response may have a secondary function in the development of muscles and the muscle contraction apparatus.

TNFSF

Tumor necrosis factor superfamily (TNFSF) is a large family of receptors and ligands that stimulate the immune response, apoptosis and cell survival¹²². TNFRSF11A is also known as receptor activator of NFκB ligand (RANK) and is essential to osteoclast formation and activation¹²³. However, RANK is also expressed in lungs, kidneys and skeletal muscle¹²³. RANK induces downstream expression of AKT and NFκB, and therefore is anti-apoptotic¹²³.

HTT

Huntington disease is caused by a CAG repeat expansion in the huntingtin protein (HTT). The HTT protein plays a role in suppressing apoptosis¹²⁴. Functional HTT is essential for embryonic development, and overexpression of HTT prevents cleavage of PAKs by CASP3¹²⁴. PAKs are kinases that regulate cell survival, and HTT protects cells that are exposed to apoptotic inducing signals by preventing cleavage of PAKs¹²⁴.

Transcription Factor Genes

STAT1 and STAT4

Signal transducers and activators of transcription (STAT) proteins are transcription factors that regulate several cellular processes including cell proliferation, apoptosis, differentiation and angiogenesis¹²⁵. STATs are activated by the Janus tyrosine kinase (JAK) pathway, but can also be activated by an inherent tyrosine kinase activity of

growth factors¹²⁵. STAT1 “is considered a tumor suppressor since it inhibits growth and acts as a proapoptotic factor”, and can stimulate caspase expression¹²⁵.

ATF2/CREB2

ATF2 is a member of the leucine zipper activator protein 1 (AP-1) transcription factor family¹²⁶. These proteins can form homo- or heterodimers between family members to stimulate gene expression¹²⁶. ATF2 is activated by the JNK pathway and is upregulated as is c-Jun, of which a majority heterodimerize¹²⁶. The c-Jun/ATF2 heterodimer leads to apoptosis in response to cellular stress, and when this complex is blocked, apoptosis is inhibited¹²⁶. Homodimers of ATF2 can not lead to apoptosis, nor can an overexpression of c-Jun cause cell death, suggesting that ATF can only induce apoptosis when bound to c-Jun¹²⁶.

NFAT2

Nuclear factor of activated T-cells (NFAT) is a family of calcium dependent transcription factors¹²⁷. NFAT2 is expressed in most cells involved in the immune response, but is also expressed in other cells, although not ubiquitously expressed¹²⁷. NFAT2 has been shown to regulate skeletal muscle through control of myocyte differentiation¹²⁷. In addition, NFAT2 stimulates apoptosis through several pathways including the FAS ligand and glucocorticoid induced apoptosis¹²⁷.

CREB1

CREB (c-AMP dependent response element binding protein) is activated along with AP-1 genes and the JNK pathway in response to oxidative stress and regulates genes involved in cellular proliferation and apoptosis¹²⁸. CREB recruits c-AMP and other cofactors to CRE binding sites, which are necessary for activation of target genes

including c-FOS and BCL-2¹²⁸. CREB has been shown to downregulate production of BCL-2, and inhibition of CREB results in an increase in BCL-2 transcription and cell survival¹²⁸. Therefore, CREB1 is a pro-apoptotic protein.

TFDP1/2

Transcription factor DP2 (TFDP2) dimerizes with E2F family members and increases transcription of target genes including those involved in apoptosis and the cell cycle¹²⁹. Targets of DP2 include *ARF*, *ATM*, *CASP8*, *CASP3/7*, *CASP9* and *BAK/BAD* (pro-apoptotic members of the BCL-2 family)¹³⁰. The p53 mediated apoptotic pathway is activated by DP proteins, and this cascade stimulates the mitochondrial mediated apoptotic pathway¹³⁰.

RBAK

Retinoblastoma-associated Kruppel-associated box protein (RBAK) is a transcription factor that is widely expressed in embryonic and adult tissues¹³¹. RBAK and acts as a transcriptional repressor by dimerizing with E2F proteins. It can also stop the cell from progressing to the synthesis stage of the cell cycle¹³¹. Many of the E2F targets are proapoptotic genes (see TFDP1/2) which can be repressed by RBAK¹³¹.

Engulfment of Apoptotic Body Genes

EMP

Erythroblast macrophage protein (EMP) is expressed in both erythroblasts and macrophages and helps adhesion between these two cell types during blood production and in the fetal liver¹³². EMP is required for normal differentiation of erythroblasts, and targeted disruption of this protein leads to an embryonic lethal mouse¹³². When

erythroblasts are cultured in the presence of an anti-EMP antibody a six-fold increase in apoptosis was seen¹³².

GULP1

Rapid processing of apoptotic bodies is important during embryogenesis¹³³. GULP1 acts in the engulfment of apoptotic bodies¹³³. GULP1 is highly conserved, and in fact can rescue *c. elegans* deficient in the GULP1 homolog CED-6¹³³. GULP1 also interacts directly with low density lipoprotein-related protein 1 (LRP-1) and multiple epidermal growth factor domains 10 (MEGF10), which are also involved in engulfing apoptotic bodies¹³³.

Other Apoptosis Related Genes

ING1

Inhibitor of growth (ING) proteins are a family of highly conserved apoptotic proteins¹³⁴. ING1 and ING3 both stimulate apoptosis, but through separate arms of the apoptotic pathway. ING1 inhibits PCNA, which inhibits apoptosis and activates p53¹³⁴. ING1 mice knockouts show increased apoptosis in response to radiation, which suggests that ING1 has an anti-apoptotic role in response to DNA damage¹³⁴. However, during embryogenesis in *Xenopus laevis* ING1 has a proapoptotic expression pattern¹³⁴.

ATR

Ataxia-Telangiectasia Mutated and Rad-3 related (ATR) is in the DNA damage pathway, and has an anti-apoptotic effect in response to this damage¹³⁵. ATR activates Chk1, which prolongs activity at the replication forks and restarts replication forks where activity has been aborted¹³⁵. The ATR pathway leads to inhibition of apoptosis, and cells

depleted of Chk1 activate CASP3¹³⁵. ATR also phosphorylates and activates p53, leading to apoptosis¹³⁶.

SOCS6

Suppressors of cytokine signaling (SOCS) block signaling of cytokines, “a large family of secreted glycoproteins that regulate fundamental biological processes, including embryonic development, immunity, and haematopoiesis”¹³⁷. Little is known about the function of SOCS6, but family members SOCS1-3 have been well characterized. SOCS1 deficient mice develop normally but have a low lymphocyte count due to an inhibition of anti-apoptotic BCL-2 family member BAX¹³⁷. In addition, SOCS1 acts in a feedback loop by inhibiting cytokine signaling¹³⁷.

CERKL

Ceramide-kinase like (CERKL) is involved in the metabolism of ceramide, a sphingolipid¹³⁸. Sphingolipids are lipid messengers that act as cell sensors produced in response to cell stress, cytokines, and cytotoxins and initiate apoptosis¹³⁸. However, anti-apoptotic signals can inhibit apoptosis by removing ceramide from the cell through phosphorylation by a ceramide kinase such as CERKL.

WDR19

Little is known about WDR19, but expression has been found in several expression arrays. One expression profile found that WDR19 is expressed in the quadriceps, although at lower levels than in extraocular muscle¹³⁹. Apoptosis induced by overexpression of E2-F1 and treatment with doxyrubicin, increased expression of WDR19 in melanoma cells¹⁴⁰.

Apoptotic genes play an important role in limb and muscle morphogenesis. All of the genes that encode proteins discussed above are involved in some part of the apoptotic pathway, and all are located in the clubfoot deletion regions. Interestingly, many of the genes involved in this pathway interact through NF κ B, which has been shown to be involved in muscle development and critical to the immune response (Fig 4.1)¹⁴¹. Because these genes could potentially perturb muscle development during embryogenesis, this study was undertaken to determine whether genetic variation in these candidate genes is associated with isolated clubfoot.

Methods

Sample Collection

This study was approved by the University of Texas Committee for Protection of Human Subjects (HSC-MS-04-239) and the IRBs of all participating centers. Probands with clubfoot were identified through Shriners Hospitals for Children of Houston and Los Angeles, University of Vancouver, British Columbia and the Scottish Rite Hospital of Dallas. Individuals with chromosomal abnormalities, syndromes or postnatal events associated with clubfoot were excluded. Only cases of isolated clubfoot and their family members were included in the study. Ethnicity was self-reported. The sample was composed of 304 simplex trios, of which 124 were non-Hispanic white and 180 were Hispanic, and 179 multiplex families, of which 105 were non-Hispanic white and 74 Hispanic.

DNA Extraction and Genotyping

DNA was extracted from blood and/or saliva samples collected from all individuals. DNA was extracted from blood using the DNA Isolation Kit for Mammalian Blood (Roche, Palo Alto, CA) or saliva with Oragene Purifier (DNA Genotek Inc., Ottawa, Ontario, Canada). Samples were genotyped using SNPlex™ Genotyping System following the manufacturer's protocol (Applied Biosystems, Foster City, CA) and analyzed on a 3730 DNA Analyzer using GeneMapper® 4.0 (Applied Biosystems, Foster City, CA).

Data Analysis

Several software programs were used to test for association including Pedigree Disequilibrium test (PDT) and Association in the Presence of Linkage (APL)^{91,142}. The Geno-PDT, a modified version of the PDT, was employed to test for specific genotypes when considering dominant or recessive models⁷⁵. Altered transmission of pairwise haplotypes within a gene was determined using APL. SNPs were then evaluated for gene-gene interactions using Generalized Estimating Equations (GEE)⁹².

Results

One hundred ninety-two SNPs in 29 apoptotic genes were genotyped in 304 simplex simplex (124 NHW and 180 Hispanic) and 179 multiplex families (105 NHW and 74 Hispanic). All SNPs and locations are shown in Appendix B, Table B1. Call rates were lower than those found in previous genotyping studies using Taqman assays. Genotyping was performed using ABI SNPlex, which often produces varying results. For some of the SNPs, some data plots required manual calls and for others genotypes could not be

determined. Fifty-six of the SNPs were out of HWE, which is most likely related to the low call rates. These SNPs were excluded from further analysis resulting in 165 SNPs analyzed in the NHW group and 157 SNPs in the Hispanic group.

Significant associations were found in the single SNP analysis with little overlap between Hispanics and NHW groups (Table 4.2). For the NHW group, the most significant finding was found by all three tests for rs4690055 in *TNIP2* in the simplex families (Table 4.2A). *TNIP2* functions as an inhibitor of NF κ B as does *TLR10*. rs11096957, in *TLR10*, is a nonsynonymous coding SNP (N241H) that was significant in simplex (p=0.003) and in the aggregate (p=0.004) groups. *TNFSF13B* stimulates apoptosis through *CASP3*, although it can also activate *NFKB* (Fig 4.1). Interestingly, rs9520835 in *TNFSF13B* was significant in the aggregate NHW group (APL p=0.004 and PDT p=0.009) and in the multiplex families (p=0.005). Altered transmission was also found for a SNP in *CREB1*, *TRIAD3*, *GULP1* and *EMP/MAEA* genes.

In the Hispanic group, four SNPs in the *NFATC1* (rs8097537, rs3894049, rs12608349 and rs370989) showed altered transmission (Table 4.2B). *NFATC1* is responsible for activating the apoptotic pathway through the FAS receptor (Fig 4.1). Two of the four significant *NFATC1* SNPs, rs8097537 (p=0.0004) and rs3894049 (p=0.0002) were identified by all three analytic programs. Altered transmission in the aggregate and simplex groups was found for all four *NFATC1* SNPs. However, rs370989 was also significant in the multiplex families (p=0.001). FAS can also activate NF κ B through Casp10 and the MAP kinase pathway (Fig 4.1). *ZAK* also activates NF κ B through the MAP kinase pathway. Altered transmission of rs11686011 in the *ZAK* gene was found for the aggregate group (p=0.007), but not when stratified by family history. Altered

Table 4.2. Apoptosis genes: Single SNP results

A. NHW

Families	SNP	Gene	Gene Loc.	APL	PDT	GenoPDT
Combined	rs11096957	<i>TLR10</i>	E4 N241H	0.004	0.041	0.148
	rs9520835	<i>TNFSF13B</i>	I3	0.004	0.009	0.023
Multiplex	rs10932201	<i>CREB1</i>	I4	0.005	0.137	0.081
	rs9520835	<i>TNFSF13B</i>	I3	0.005	0.113	0.166
	rs11771172	<i>TRIAD3</i>	I2	0.006	0.336	0.361
Simplex	rs6724428	<i>GULP1</i>	I4	0.006	0.025	0.112
	rs1316393	<i>EMP/MAEA</i>	I3	0.010	0.063	0.114
	rs11096957	<i>TLR10</i>	E4 N241H	0.003	0.090	0.203
	rs4690055	<i>TNIP2</i>	I2	0.004	0.001	0.007

B. Hispanic

Families	SNP	Gene	Gene Loc.	APL	PDT	GenoPDT	
Combined	rs10930693	<i>CREB2</i>	I3	0.038	0.005	0.013	
	rs12608349	<i>NFATC1/NFAT2</i>	I4	0.102	0.061	0.001	
	rs370989	<i>NFATC1/NFAT2</i>	I10	0.003	0.003	0.003	
	rs3894049	<i>NFATC1/NFAT2</i>	I10	0.0002	0.001	0.004	
	rs8097537	<i>NFATC1/NFAT2</i>	I3	0.025	0.0004	0.002	
	rs2280233	<i>STAT1</i>	I14	0.155	0.007	0.040	
	rs6751855	<i>STAT1</i>	U	0.144	0.491	0.010	
	rs7239261	<i>TNFRSF11A</i>	I1	0.007	0.075	0.179	
	rs8094884	<i>TNFRSF11A</i>	I6	0.071	0.010	0.111	
	rs852374	<i>TRIAD3</i>	D	0.005	0.003	0.020	
	rs11686011	<i>ZAK</i>	I14	0.007	0.182	0.299	
	Multiplex	rs6446723	<i>HTT</i>	I10	0.0004	0.330	0.587
		rs370989	<i>NFATC1/NFAT2</i>	I10	0.001	0.274	0.164
rs4941125		<i>TNFRSF11A</i>	I1	0.0004	0.608	0.810	
rs852522		<i>TRIAD3</i>	I13	0.006	0.017	0.116	
Simplex	rs10930693	<i>CREB2</i>	I3	0.024	0.004	0.006	
	rs12608349	<i>NFATC1/NFAT2</i>	I4	0.021	0.016	0.005	
	rs370989	<i>NFATC1/NFAT2</i>	I10	0.077	0.003	0.009	
	rs3894049	<i>NFATC1/NFAT2</i>	I10	0.007	0.0001	0.0003	
	rs8097537	<i>NFATC1/NFAT2</i>	I3	0.053	0.0003	0.001	
	rs13065446	<i>TFDP2</i>	U	0.369	0.194	0.009	

Gene Loc = gene location: I = intron, D = downstream, U = upstream,

transmission was also found for one or two SNPs in *CREB2*, *STAT1*, *TNFRSF11A*, *TRIAD3*, *HTT* and *TFDP2* genes.

While overall there was little overlap between SNP variants detected in the NHW and Hispanic groups, there were some interesting similarities. For example, two different SNPs in *TRIAD3* showed altered transmission in both groups (Table 4.2). rs11771172 in *TRIAD3* ($p = 0.006$) was significant in the multiplex NHW families only, whereas rs852374 was significant in the aggregate and multiplex Hispanic families. This suggests that *TRIAD3* is important in both ethnic groups. *TRIAD3* inhibits NF κ B activity by inhibiting TLR. rs10932201 in *CREB1* was significantly associated in the multiplex NHW families ($p=0.005$), while rs10930693 in *CREB2* was significant in the aggregate ($p=0.005$) and simplex ($p=0.004$) Hispanic families. *CREB1* and *CREB2* are members of a gene family that function in different branches of the apoptotic pathway. It is interesting that different genes in the same pathway were significantly associated in the different ethnic groups. Similarly, two SNPs, rs7239261 and rs8094884, in *TNFRSF11A* receptor demonstrated altered transmission in the aggregate ($p=0.007$ and 0.01 , respectively) Hispanic families, whereas rs9520835, in *TNFSF13B*, a ligand family member, showed altered transmission in the aggregate ($p = 0.004/0.009$) and multiplex ($p = 0.005$) NHW families. This suggests that the same genetic pathway is being perturbed in different ethnic groups but by variation in different members of the same gene family.

Seven two-SNP haplotypes in five genes in the NHW group and 28 two-SNP haplotypes in five different genes in the Hispanic group found (Table 4.3). The most significant haplotype in the NHW group involved rs1451821 and rs3733280 in *WDR19* ($p=0.002$). Interestingly, most haplotypes in the NHW group do not include significant

variants from the single-SNP analysis. For example, only one significant haplotype, contained a SNP, rs6724428 (*GULP1*), that showed altered transmission in the single-SNP analysis (Table 4.3A, $p=0.005$). However, three significant haplotypes were identified for *GULP1*. rs10931359 (*GULP1*) was involved in two haplotypes with rs7593946 ($p=0.009$) and rs12624002 ($p=0.004$).

In contrast, for the Hispanic group, 25 of the 28 significant two-SNP haplotypes contained SNPs that showed significantly altered transmission in the single-SNP analysis (Tables 4.3B). However, the most significant haplotypes were found for SNPs in *TRIAD3* and *NFAT2*. rs852374 showed altered transmission with seven other *TRIAD3* SNPs and rs3894049 with nine SNPs in *NFAT2*. This may be due to linkage disequilibrium between the variants, and this analysis is ongoing. Interestingly, the overtransmitted allele for rs3894049 in most of the haplotypes is allele 2, but in the rs4799055/rs3894049 haplotype the overtransmitted allele for rs3894049 is allele 1, which is the opposite of all the other haplotypes ($p=0.001$). The significant SNP haplotypes for *WDR19* and *CARD11* did not include the SNPs identified in the single SNP analysis.

Twenty-two gene-gene interactions were identified and there was no overlap when comparing by ethnicity (Table 4.4). For the NHW group, the most significant gene interactions were between rs6769676 in *RNF7* and rs4273389 in *ATR* ($p=0.0001$) and rs1517352 in *STAT4* and rs11096957 in *TLR10* ($p=0.0001$). The most significant interactions in the Hispanic group were between rs4675272 in *SUMO1* and rs9520835 in *TNFSF13B* ($p=0.0002$), rs925847 in *STAT4* and rs8094884 in *TNFRSF11A* ($p=0.0003$), and rs4972533 in *ZAK* and rs3779092 in *TRIAD3* ($p=0.0003$). Interestingly, *TNFSF13B*

Table 4.3. Two-SNP Haplotypes Results

A. NHW

Gene	SNP 1	SNP 2	p-value	OT	UT
<i>ATR</i>	rs6440085	rs6792259	0.003	22	-
	rs7593546	rs10931359	0.009	21	-
<i>GULP1</i>	rs12624002	rs10931359	0.004	11	12
	rs6724428	rs1354905	0.005	12	11
<i>ING1</i>	rs1441043	rs6492308	0.005	21	-
<i>WDR19</i>	rs1451821	rs3733280	0.002	21	12
<i>ZAK</i>	rs4344898	rs1837470	0.005	11	22

B. Hispanics

Gene	SNP 1	SNP 2	p-value	OT	UT
<i>TNFRSF11A</i>	rs4941125	rs7239261	0.008	-	11
	rs7239261	rs8094884	0.003	22	11
	rs7239261	rs2290154	0.007	22	12
<i>TRIAD3</i>	rs852374	rs1468996	0.008	11, 12	21, 22
	rs852374	rs2302907	0.007	11, 12	21, 22
	rs852374	rs13239194	0.009	11	21
	rs852374	rs11771172	0.003	11	21
	rs852374	rs6971918	0.005	11	21
	rs852374	rs10257204	0.003	12	-
	rs852374	rs6967635	0.009	11	21
<i>WDR19</i>	rs1451821	rs9997015	0.006	21	11
	rs3733280	rs1057807	0.009	11, 12	21
<i>CARD11</i>	rs1713911	rs7778444	0.007	11, 21	22
<i>NFAT2</i>	rs8097537	rs12608349	0.007	21	11
	rs9962479	rs12608349	0.002	22	-
	rs12608349	rs370989	0.004	11	12
	rs4799055	rs370989	0.005	21, 11	22, 12
	rs7227107	rs370989	0.002	11	12
	rs12608349	rs3894049	0.001	12	11
	rs1660139	rs3894049	0.002	12	11
	rs1667673	rs3894049	0.006	22, 12	21
	rs177820	rs3894049	0.004	22, 12	11, 21
	rs2036892	rs3894049	0.004	22	21
	rs370989	rs3894049	0.002	12	21
	rs4799055	rs3894049	0.001	11, 21	22, 12
	rs7227107	rs3894049	0.0004	12	11
	rs8090692	rs3894049	0.002	22	11
rs8097537	rs7227107	0.007	11	-	

Table 4.4. Gene interactions

A. NHW

Gene 1	SNP 1	Gene Loc.	Gene 2	SNP 2	Gene Loc.	p-value
<i>CREB1</i>	rs2551640	I1	<i>HTT</i>	rs2285086	I2	0.0002
<i>GULP1</i>	rs1354905	I8	<i>TNFSF13B</i>	rs10508198	I2	0.0004
					E1	
<i>RNF7</i>	rs1980191	U	<i>TLR10</i>	rs11096957	N241H	0.0008
<i>RNF7</i>	rs6769676	I1	<i>ATR</i>	rs4273389	I31	0.0001
<i>RNF7</i>	rs6769676	I1	<i>ATR</i>	rs13085998	I16	0.0004
					E1	
<i>STAT4</i>	rs1517352	I6	<i>TLR10</i>	rs11096957	N241H	0.0001
<i>WDR19</i>	rs1057807	D	<i>UBE2K</i>	rs302947	I2	0.0008
<i>ZAK</i>	rs13032010	I2	<i>CREB1</i>	rs10932201	I4	0.0007

B. Hispanics

Gene 1	SNP 1	Gene Loc.	Gene 2	SNP 2	Gene Loc.	p-value
<i>RBAK</i>	rs7778444	3'	<i>TNFRSF11A</i>	rs8089829	I7	0.0009
<i>RBAK</i>	rs7778444	3'	<i>TNFSF13B</i>	rs9514828	U	0.0008
<i>CERKL</i>	rs1047307	3'	<i>NFAT2</i>	rs177820	I10	0.0009
<i>CERKL</i>	rs1866888	U	<i>HTT</i>	rs362331	I41	0.0007
<i>GULP1</i>	rs12474692	I2	<i>RBAK</i>	rs7778444	3'	0.0003
<i>GULP1</i>	rs7586390	I1	<i>RBAK</i>	rs7778444	3'	0.0006
<i>RNF7</i>	rs6776205	D	<i>TRIAD3</i>	rs13247447	I13	0.0005
<i>STAT1</i>	rs12468579	D	<i>STAT4</i>	rs925847	I21	0.0008
<i>STAT4</i>	rs925847	I21	<i>TNFRSF11A</i>	rs8094884	I6	0.0003
<i>SUMO1</i>	rs4675272	I1	<i>TNFSF13B</i>	rs9520835	I3	0.0002
					E9	
<i>TFDPI</i>	rs7325214	U	<i>NFAT2</i>	rs7227107	Syn	0.0007
					E1	
<i>TRL6</i>	rs3821985	Syn	<i>UBE2K</i>	rs305827	I4	0.0005
<i>ZAK</i>	rs11686011	I14	<i>STAT4</i>	rs4341967	I3	0.0006
<i>ZAK</i>	rs4972533	I8	<i>TRIAD3</i>	rs3779092	I13	0.0003

Gene Loc.=gene location, 3'=3'UTR, U=upstream, I=intron, D=downstream, E=exon, Syn=synonymous

and *TNFRSR11A* are both *TNF* family members suggesting that perturbation of this branch of the apoptotic pathway may play role in clubfoot in Hispanics.

Many of the variants identified in single SNP analysis and in the gene-gene interactions were located in potential transcription factor binding sites (Table 4.5). Three prediction programs were used to determine if there were differences in transcription factor binding sites between ancestral and alternate alleles for the variants. Each of the programs predicted that most of the SNPs create or ablate transcription factor binding sites, although the programs did not agree on which sites. Interestingly, two prediction programs agreed on the creation of an H4TF-2 site for rs4941125 in *TNFRSF11A*. Also of note, a retinoic acid receptor binding site is predicted to be lost for rs4675272 in *SUMO1*. This is important because of the role retinoic acid plays in the development and patterning of them limbs (Chapter 1). A STAT5b binding site is predicted to be lost for rs1047307 in *CERKL*, and this transcription factor is a family member of *STAT1* and *STAT4*, which have altered transmission in the clubfoot population of this study. Also, for rs9514828 in *TNFSF13B*, an NFκB binding site, which plays an integral role in the immune response and apoptotic pathway (Fig. 4.1). However, changes are predicted to occur, and further testing will be required to determine if the alleles are functional.

Table 4.5. Predicted transcription factor binding sites. Comparison of ancestral and alternative alleles of significant SNPs in putative regulatory regions. Ancestral allele defined by the allele present in chimpanzee. Gene Loc.=gene location, 3′=3′UTR, U=upstream, I=intron, D=downstream, E=exon, Syn=synonymous

AFP1=alpha fetoprotein enhancer binding protein 1, AKNA=AT hook transcription factor, AP1/2 α =activator protein 2, C/EBP=CCAAT enhancer binding protein, CDC2=Cell division cycle 2, c-myc=myc oncoprotein, CTCF=CCCTC binding factor, E1A-F=E1A enhancer binding protein, EG=epsilon globin, EGFR=epidermal growth factor, GATA1=GATA binding protein 1, GH=growth hormone, GM-CSF=granulocyte/macrophage colony stimulating factor, GR=glucocorticoid receptor, H4TF-2=Histone 4 pHu4A gene, HNF3=hepatocyte nuclear factor 3, HSTF=heat shock transcription factor, ICSBP=interferon consensus sequence binding protein, IGH=immunoglobulin heavy chain, IL4/6=interleukin 4/6, LUN1=Lung, LyF1 =lymphoid transcription factor, MBP=Myelin basic protein, NMP-1=nuclear matrix protein, Oct-1=POU class 2 homeobox 1, Pit 1a=pituitary-specific factor 1, RAR α =retinoic acid receptor alpha, SP1=stimulating protein 1, TBP=TATA binding protein, TCF2 α =T cell factor 2 alpha, TFIID=transcription initiation factor D

SNP	Gene	Gene Loc.	Anc Allele	Alt Allele	Alibaba		Patch		TESS	
					Anc Allele	Alt Allele	Anc Allele	Alt Allele	Anc Allele	Alt Allele
rs1047307	<i>CERKL</i>	3'	G	A	HSTF	None	STAT5b	None	None	C/EBP
rs1866888	<i>CERKL</i>	U	T	C	None	None	Pit 1a	AKNA, IL6, C/EBP, MBP, EG, TFIID, TBP	CDC2, GH	AP1
rs2551640	<i>CREB1</i>	I1	G*	A*	None	None	None	CDC2	None	None
rs7586390	<i>GULP1</i>	I1	A	G	None	None	AFP1	None	None	CDC2
rs7778444	<i>RBAK</i>	3'	G	A	None	Oct-1	None	None	None	None
rs1980191	<i>RNF7</i>	U	A	T	None	Pit 1a	GM-CSF	NMP-1, MBP, AKNA, EG, TFIID, TBP	None	GH
rs6769676	<i>RNF7</i>	I1	G	T	TBP	None	None	None	None	TCF2 α , E1A-F, CDC2
rs6751855	<i>STAT1</i>	U	G	A	None	ICSBP, GATA1, C/EBP	GM-CSF	c-myc, HNF3, EG, TFIID, TBP	None	None
rs4675272	<i>SUMO1</i>	I1	G	C	None	None	GR, RAR α 1	None	None	None
rs7325214	<i>TFDP1</i>	U	C*	T*	Oct-1	None	None	LyF1, AP2 α , LUN1	None	SP1, c-myc
rs13065446	<i>TFDP2</i>	U	C	T	None	None	CTCF, EGFR, IGH	Pit 1a	C/EBP	GH
rs4941125	<i>TNFRSF11A</i>	I1	A	G	None	C/EBP, SP1	IL4	H4TF-2	None	HINFP
rs7239261	<i>TNFRSF11A</i>	I1	C	A	None	None	None	None	None	C/EBP
rs9514828	<i>TNFSF13B</i>	U	C	T	None	NF κ B	None	None	None	None

Discussion

In previous studies, we found an association between mitochondrial mediated apoptotic genes and isolated clubfoot^{78,106}. For this reason and because clubfoot chromosomal deletion regions contained many apoptotic genes, 192 flanking and intragenic SNPs in 29 apoptotic genes were interrogated. For the NHW group, the most significant association was found for SNP rs11096957 in *TLR10*, which creates an amino acid substitution. The H241N is on the surface of the protein, and is not predicted to be deleterious to the function of the protein (www.snp3d.org). However, this variant may still be functional if it disrupts splicing enhancers or silencing sites. This SNP may also be in linkage disequilibrium with other variants that may be functional.

In the Hispanic group, the most significant SNP associations were found for *NFAT2* and *TNFRSF11A* in the single SNP analysis. Although these *NFAT2* SNPs were in introns, they were not located in the first or second introns that can harbor transcription factor binding sites. There are multiple transcripts of *NFAT2*, and isoform D has its start site in exon 3, and all SNPs are located downstream of this start site. Therefore, these particular variants are not likely to be involved in promoter regulation. However, these SNPs could affect splice site regulatory elements or be in LD with other functional variants. rs4941125 in *TNFRSF11A* is in the first intron and could potentially be located in a transcription factor binding site. Both Patch and TESS prediction programs expect a HINFP (Histone 4 transcription factor) binding site to be created in the individuals who have the alternate allele compared to the ancestral allele (Table 4.5). H4TF-2 is a transcription factor only expressed during DNA synthesis¹⁴³. The creation of this binding

site in *TNFRSF11A* may lead to abnormal production and activation of the TNF receptor. This finding may be particularly important for the Hispanic group.

Interestingly, the two-SNP haplotypes in the NHW did not include any of the SNPs identified in the single SNP analysis. The opposite was found for the Hispanic group, where many significant haplotypes contained SNPs identified in the single analysis such as rs3894049 in *NFAT2*. Together these results suggest that there are distinct genetic etiologies for NHW and Hispanics, and different genes are involved in clubfoot for each population. The Hispanic haplotypes confirm the results of the single SNP analysis, suggesting that *NFAT2* and *TRIAD3* are important in clubfoot development within the Hispanic population. Both of these genes act at the cell membrane level of the apoptotic cascade and perturbation of gene expression may have a global effect on the apoptotic pathway and downstream proteins.

Many of the associated SNPs may perturb gene expression, and functional testing is required to determine if the predicted transcription factor binding sites change expression. Interestingly, although there was no overlap of SNPs involved in gene-gene interactions between NHW and Hispanic groups, many of the same genes were involved in the interactions. This suggests that while there is allelic heterogeneity between families, a small portion of the same genes consistently contribute to clubfoot. However, even though the same genes are involved in gene-gene interactions, there were significant differences between the variants involved in the interactions in NHW and Hispanics. This supports the complex model for clubfoot in which perturbation of many genes and environment factors contribute to clubfoot.

Limb formation is a complex process involving cell proliferation and patterning, programmed cell death, and correct positioning of the limb^{21,22}. The hind limb begins as a small swelling during the fourth week of gestation and morphogenesis is complete by the end of the eighth week²³. However, the legs, which develop in the midline in equinus, should move to plantar grade by the twelfth week²⁴. Failure of this final movement results in clubfoot. Perturbations in any of the developmental processes may affect the final foot positioning, as would abnormal muscle or tendon development that is universally associated with clubfoot^{1,7,11,14}. It is unclear whether the abnormal musculature in clubfoot is a primary or secondary abnormality. It has recently been shown that apoptosis is required for development of the muscle bellies and correct attachment to tendons²². Disruption of the apoptotic pathway, whether by increased or decreased activity, could alter morphogenesis and prevent the fetal limb movement required for normal foot development.

Based on our model shown in Fig 4.2, individual genetic variants associated with clubfoot may be necessary but not sufficient to cause the disorder. For the individual, the combination of many variations within different causative genes, e.g. a high risk genetic haplotype (but not necessarily within one gene), could contribute to clubfoot. In addition, those individuals with specific high-risk haplotypes may be more susceptible to environmental exposures. For example, maternal smoking in the general population increases the risk of clubfoot four fold, however maternal smoking in women with a family history of clubfoot have a twenty fold increased risk of clubfoot⁵⁸. When this information is incorporated into the working model of clubfoot in Fig. 4.2, maternal smoking is more likely to contribute to clubfoot if the babies and/or mothers also have

the high-risk haplotype. This model of clubfoot etiology incorporates genetic variants in different pathways including xenobiotic and toxin metabolism, limb and muscle development, and apoptosis which have all recently been associated with clubfoot (Fig. 4.2)^{78,106,144}. There may be a threshold of genetic variants needed to cause clubfoot, with greater number of high-risk variants increasing the severity of clubfoot. Our findings fit this model, which is based on the multifactorial model first proposed by Carter et al. in 1965¹⁴⁵. Determining all of these high-risk haplotypes is the continuing challenge but will improve genetic counseling and provide more precise risk assessments.

This study had a limitation in that the call rates of many of the variants that were interrogated were lower than expected. This is most likely due to the chemistry of the genotyping assay, SNPlex. The significant results reported here should be replicated with a higher fidelity assay, such as TaqMan in the primary data set and then validated in a secondary population such as the recently collected case-control (N=750) clubfoot population.

In summary, this study interrogated apoptotic genes identified in clubfoot deletion regions. We found significantly associated SNPS in genes that are different between ethnic groups yet function in many similar apoptotic pathways. This provides a degree of confidence in our results, which nevertheless require validation. These results add to our growing body of knowledge about etiologic causes of clubfoot.

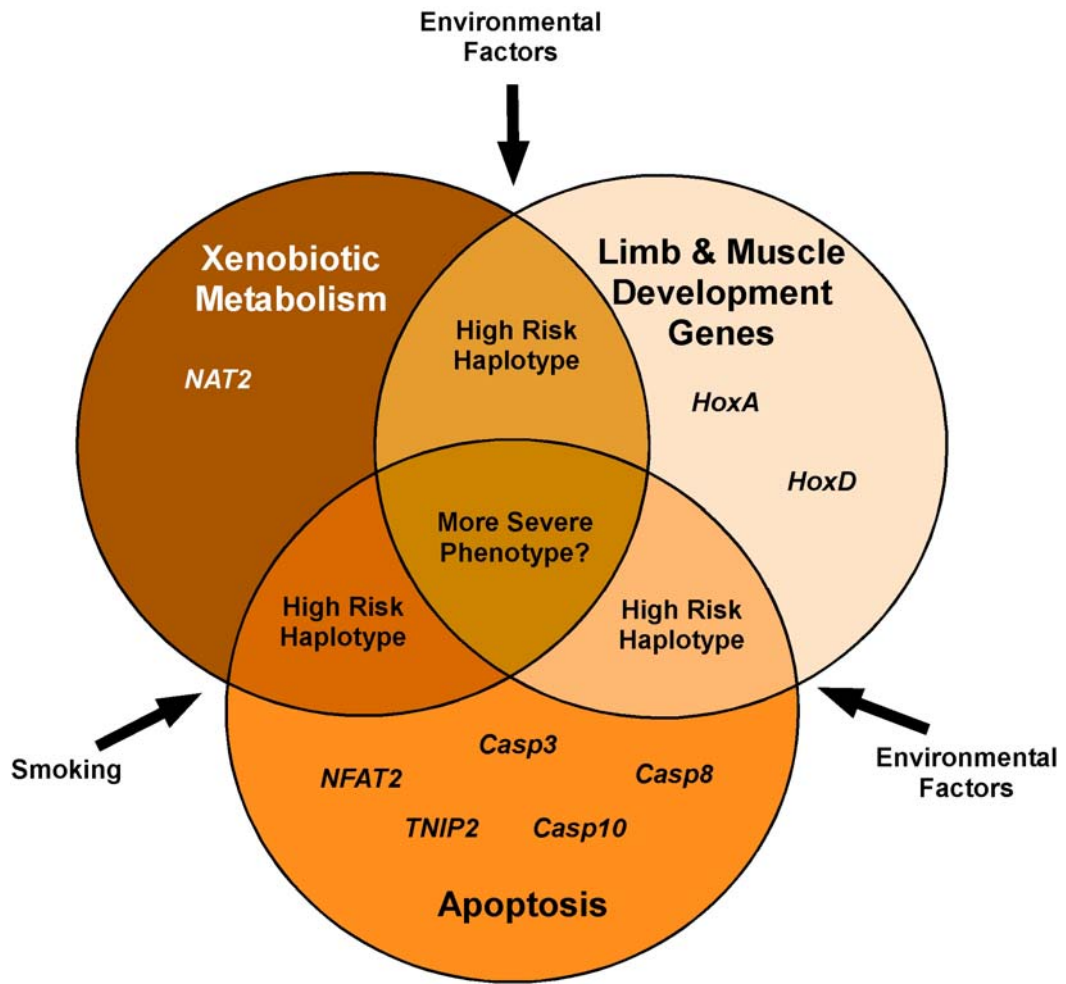


Fig 4.2. Clubfoot Model

Chapter 5: HOX Transcription Factors and Clubfoot

This chapter is a presentation of work from a previously published paper. For more information please refer to: Ester, AR, Weymouth, K, et al. Altered transmission of *HOX* and apoptotic SNPs identify a potential common pathway for clubfoot. American Journal of Medical Genetics. December, 2009. 149A (12): 2745-52.

HOX Genes

The *HOXD* family of genes is a group of candidate genes, as they map to chromosome 2q31-33, a deletion region identified by Brewer et al. to be associated with clubfoot^{69,144}. The *HOX* proteins are transcription factors that direct patterning of the axial skeleton and muscle and limb development^{31,32}. The *HOX* gene family consists of 39 genes in four paralogous clusters (A, B, C and D) on different chromosomes (7p15.3, 17q21.3, 12q13.3 and 2q31.1, respectively). The *HOX* clusters share common genomic structure (Fig. 1) and are temporally and spatially regulated and expressed during embryogenesis^{31,33}. “Expression of the 3’ genes of the clusters begins in the superior regions of the embryo and progresses more inferiorly as the more 5’ genes in the cluster are expressed in the developing embryo”¹⁴⁶. For example, *HOXD9*, located towards the 5’ end of the cluster, is expressed proximally as the limb bud develops. The further 5’ genes are, the later and more distal they are expressed as the limb extends and differentiates (Fig. 5.1)³¹.

The 5’ *HOXA* genes are expressed in fore- and hind limb muscles of, and are involved in patterning and differentiation of muscles in both embryonic limbs and adult limbs during muscle repair³⁴. These 5’ genes have also been detected in myoblast and myotube cell lines³⁴. *HOXA11* and *HOXA13* have been shown to contribute to cartilage development, and *HOXA13* regulates cell adhesion during the condensation step of skeletogenesis¹⁴⁷. The synchronized development of muscles, tendons and cartilage is crucial for proper formation of the limb²¹, and 5’ *HOXA* genes regulate each of these functions^{34,147}.

Since the *HOXA* and *HOXD* genes play important roles in normal limb development, it would be expected that mutations in these genes might disrupt normal limb development (OMIM). In fact, there are several known mutations in *HOX* genes that cause limb

malformations. The *HOXD10* T956A/M319K mutation causes autosomal dominant congenital vertical talus (CVT)^{148,149}. Interestingly, a family with CVT and the T956A/M319K mutation had one affected individual with CVT in one foot and clubfoot in the other foot¹⁴⁸. Further evidence of *HOXD10* in limb malformations comes from mice in which a deletion of the gene results in stiff hind limbs with occasional abnormal rotation of the hind leg^{150,151}. Mutations in several other *HOX* genes also cause mammalian limb abnormalities. Mutations in *HOXD13* cause human synpolydactyly^{148,152,153}. Mutations in *HOXA13* in the mouse lead to hypodactyly and the homozygous knock out of this gene is an embryonic lethal¹⁵⁴.

HOXA and *HOXD* genes are important in limb morphogenesis, and disruption of these genes individually gives rise to syndromes associated with limb abnormalities. “While few of these syndromes include clubfoot, it is unknown whether different types of genetic variation may produce a related phenotype such as isolated clubfoot”. This phenomenon has been documented in nonsyndromic cleft lip and palate, where an *IRF6* promoter variant may be etiologic in 18% of cases, whereas coding mutations cause van der Woude syndrome¹⁵⁵. Given the role of *HOX* genes in limb development, they are candidates for playing an etiologic role in isolated clubfoot. This study was undertaken to determine whether genetic variation in the *HOXA* or *HOXD* genes is associated with isolated clubfoot, to further evaluate whether the T956A/M319K *HOXD10* mutation causes clubfoot and to determine if variation associated with clubfoot affects the amount of *HOX* protein produced.

Study Design

Nine *HOXA* and 11 *HOXD* SNPs (Fig. 5.1, Table 5.1) were genotyped in a primary discovery population composed of 304 simplex trios (124 Non-Hispanic white and 180

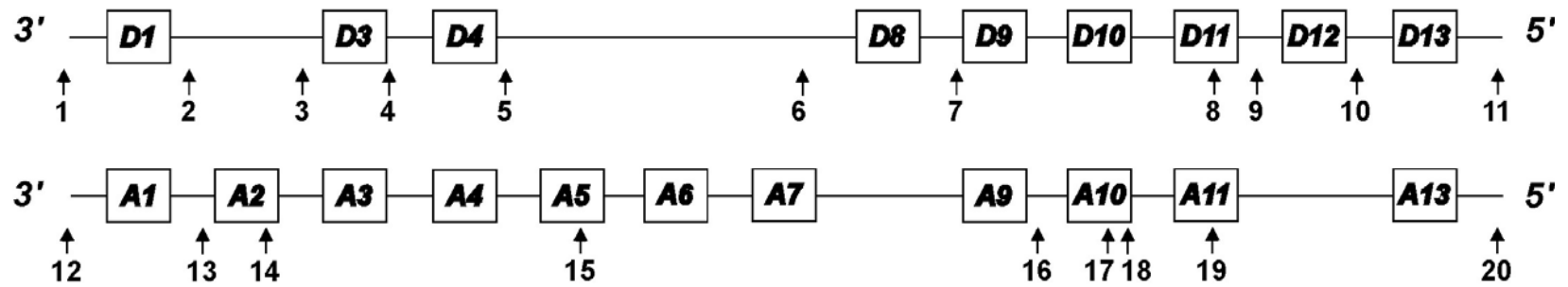
Hispanic) and 179 multiplex families (105 Non-Hispanic white and 74 Hispanic). An independent validation population consisted of 144 nonHispanic white clubfoot simplex trios.

To determine whether the T956A/M319K *HOXD10* mutation causes ITEV, 494 ITEV probands (267 nonHispanic white and 227 Hispanic) were genotyped using the TaqMan Genotyping Assay. Unrelated parents of children with sporadic nonsyndromic cleft lip and palate (NSCLP) with no known abnormalities were used for the control population consisting of 595 individuals (380 nonHispanic white and 215 Hispanic) with no anomalies. For positive controls, DNA of two affected individuals from a CVT family with the *HOXD10* T956A/M319K was used. (Ester, 2009)

Results

No T956A/M319K *HOXD10* variants were detected among either the unrelated controls. We next genotyped SNPs in the *HOXA* and *HOXD* gene clusters. All 20 examined SNPs were in HWE in both ethnic groups (data not shown). Comparisons of SNP allele frequencies between nonHispanic whites and Hispanics detected significant differences even after Bonferroni correction ($p < 0.001$) in the majority of SNPs (Table 5.1). Therefore, the data were stratified by ethnicity and examined separately. For SNPs showing no frequency differences, the data was combined across ethnicities for APL analysis. Parametric and nonparametric multipoint linkage analysis did not detect linkage in *HOXA* or *HOXD* genes (data not shown).

Figure 5.1. Genetic layout of *HoxA* and *HoxD* gene clusters



Genes denoted by rectangles, arrows indicate SNP placement

Table 5.1. *HoxA* and *HoxD* SNPs

Gene	dbSNP	Pos.	Alleles	MAF ^a	HCF ^{b,c}	Location
<i>HoxD</i>	rs6749771	SNP1	A/G	0.391	0.685	3' of <i>HOXD</i> cluster
	rs1446575	SNP2	C/A	0.385	0.697	<i>HOXD3-HOXD1</i>
	rs1318778	SNP3	G/C	0.289	0.145	3' of <i>HOXD3</i>
	rs1542180	SNP4	G/A	0.378	0.692	5' of <i>HOXD3</i>
	rs1867863	SNP5	C/A	0.341	0.606	5' of <i>HOXD4</i>
	rs2113563	SNP6	T/C	0.306	0.257	<i>HOXD8-HOXD4</i>
	rs2592394	SNP7	C/T	0.305	0.182	<i>HOXD9-HOXD8</i>
	rs847146	SNP8	G/T	0.354	0.361	<i>HOXD11</i> (intronic)
	rs741610	SNP9	G/A	0.316	0.443	<i>HOXD12-HOXD11</i>
	rs711812	SNP10	T/G	0.325	0.438	5' of <i>HOXD12</i>
	rs6758117	SNP11	T/C	0.302	0.594	5' of <i>HOXD</i> cluster
<i>HoxA</i>	rs2462907	SNP12	C/T	0.491	0.461	3' of <i>HOXA</i> cluster
	rs6668	SNP13	C/T	0.375	0.413	3' of <i>HOXA2</i>
	rs2428431	SNP14	C/G	0.367	0.409	<i>HOXA2</i> (intronic)
	rs3757640	SNP15	C/T	0.267	0.415	<i>HOXA5</i> (intronic)
	rs3801776	SNP16	G/A	0.226	0.315	5' of <i>HOXA9</i>
	rs3779456	SNP17	T/C	0.442	0.540	<i>HOXA10</i> (intronic)
	rs1859164	SNP18	T/C	0.479	0.386	<i>HOXA10</i> (intronic)
	rs6968828	SNP19	G/T	0.462	0.445	<i>HOXA11</i> (intronic)
	rs3807598	SNP20	C/G	0.451	0.305	5' of <i>HOXA</i> cluster

^a MAF = Minor allele frequency in nonHispanic white population

^b HCF = Corresponding frequency in Hispanic of nonHispanic white minor allele

^c HCF significantly different from MAF (p<0.001) in bold

APL analysis found significant evidence for association with SNPs in both *HOXA* and

Table 5.2. Results of APL analysis of *HoxA* and *HoxD* in discovery population^{a,b}

	dbSNP	Pos.	All			Multiplex		Simplex	
			All Ethn.	NHW	Hisp.	NHW	Hisp.	NHW	Hisp.
<i>HOXD</i>	rs6749771	SNP1	— ^d	0.015	0.899	0.505	0.699	0.009	0.754
	rs1446575	SNP2	—	0.021	0.334	0.026	0.440	0.224	0.134
<i>HOXA</i>	rs6668	SNP13	0.101	0.802	0.025	0.968	0.119	0.872	0.073
	rs2428431	SNP14	0.006	0.345	0.008	0.158	0.081	0.876	0.043
	rs3801776	SNP16	—	0.004	0.143	0.032	0.114	0.046	0.449
	rs3779456	SNP17	—	0.179	0.845	0.042	0.459	0.980	0.702

^aSNPs with p<0.05 shown in bold

HOXD in the discovery sample (Table 5.2). The strongest associations in the NHW sample was seen for SNP16, located 5' to *HOXA9*, in the combined multiplex and simplex group (p=0.004) and when stratified by family history (Table 5.2). Additionally, a strong association for SNP1 in *HOXD* was found in the NHW simplex families (p=0.009). In the Hispanic sample, the strongest association was with SNP14 (p=0.008) in the *HOXA* gene cluster, with no evidence for association found with SNPs in *HOXD*. Interestingly, the combined ethnicity data for SNP14 was still significant (Table 5.2).

Significantly overtransmitted haplotypes for *HOXA* and *HOXD* were seen in NHW and *HOXA* in Hispanics (Table 5.3 and Appendix C Tables C5 - C7). “These haplotypes primarily involve the SNPs identified in the single SNP association analyses”. Among NHW the most significant haplotype for *HOXA* involves SNP16 and SNP18, which are in the 5' region of the *HOXA* cluster (Table 5.3).

Table 5.3. Overtransmitted *HoxA* and *HoxD* haplotypes in discovery sample^{a,b}

	Haplotype	p-value
NonHispanic White		
	SNP1/SNP2	0.007
<i>HOXD</i>	SNP1/SNP6	0.007
	SNP1/SNP7	0.006
<i>HOXA</i>	SNP16/SNP18	0.004
Hispanic		
	SNP14/SNP15	0.009
<i>HOXA</i>	SNP14/SNP19	0.006
	SNP16/SNP12	0.006

^aHaplotypes with p<0.01 shown

^bp-values uncorrected for multiple testing

Both of these genes are expressed during limb development. In Hispanics, haplotypes involving SNP14 or SNP16 (*HOXA*) were also overtransmitted ($0.006 \leq p \leq 0.009$; Table 5.3 and Appendix C, Table C5).

There was no evidence for gene-gene interactions between *HOXA* and *HOXD* in the NHW discovery population. However for the Hispanic population, there were interactions between *HOXA* and *HOXD* for SNPs 11/14 and 5/16 ($p=0.006$ and $p=0.007$) (data not shown).

The validation sample provided moderate evidence for association with SNP16 in *HOXA* ($p=0.028$). Analysis of 2-SNP haplotypes in the validation population identified seven *HOXA* haplotypes with altered transmission, although these haplotypes were not identified in the discovery sample (Table 5.3 and Appendix C, Table C8). “The most significant haplotypes in the validation population were SNP17/20 ($p=0.007$) and SNP18/20 ($p=0.0003$)”.

There were no interactions between SNPs in *HOXA* and *HOXD* in the validation sample (data not shown). However, multiple interactions between *HOXA* and *HOXD* SNPs were identified in the validation sample.

Previously variants in mitochondrial mediated apoptotic genes were found to be associated with clubfoot (Chapter 3), and we evaluated our data from both studies to look for potential gene-gene interactions in the discovery sample¹⁰⁶. The results are presented in Table 5.4 and Appendix C, Tables C12 and C13. While multiple interactions between *HOX* genes and mitochondrial mediated genes in both ethnicities, none of these interactions with $p < 0.01$ were the same in both ethnicities. There was, however, strong evidence for

Table 5.4. Gene-gene interactions for *Hox*, *IGFBP3* and mitochondrial-mediated apoptotic variants^{a,b}

A. nonHispanic White

Gene 1	SNP 1	Gene 2	SNP 2	p-value
<i>CASP3</i>	rs1049216	<i>HOXD</i>	rs6749771 (SNP1)	0.002
<i>CASP3</i>	rs1405937		rs6749771 (SNP1)	0.005
<i>CASP3</i>	rs1049253		rs2592394 (SNP7)	0.001
<i>CASP3</i>	rs1049216		rs2592394 (SNP7)	0.008
<i>BID</i>	rs8919		rs6758117 (SNP11)	0.007
<i>CASP3</i>	rs2720378	<i>HOXA</i>	rs6668 (SNP13)	0.004
<i>CASP3</i>	rs1049216		rs1859164 (SNP18)	0.004
<i>CASP3</i>	rs2720378		rs1859164 (SNP18)	0.004
<i>CASP10</i>	rs3900115		rs3779456 (SNP17)	0.008
<i>BID</i>	rs181405		rs3779456 (SNP17)	0.001
<i>BID</i>	rs181405		rs1859164 (SNP18)	0.005

B. Hispanic population

Gene 1	SNP 1	Gene 2	SNP 2	p-value
<i>CASP3</i>	rs4647602	<i>HOXD</i>	rs1318778 (SNP3)	0.0005
<i>CASP3</i>	rs4647602		rs2113563 (SNP6)	0.009
<i>CASP3</i>	rs2696057		rs741610 (SNP9)	0.008
<i>BCL-2</i>	rs1564483	<i>HOXA</i>	rs3801776 (SNP16)	0.002
<i>CASP9</i>	rs4233533		rs3779456 (SNP17)	0.001
<i>APAF-1</i>	rs1866477		rs6968828 (SNP19)	0.009

^aonly p<0.01 shown

^bp-values uncorrected for multiple testing

interaction between SNPs in *HOXA* and *HOXD* and SNPs in both *Casp3* and *Bid* in nonHispanic whites (Table 5.4), and in Hispanics, the strongest interactions were between SNPs in *Casp3* and *HOXD* (Table 5.4).

	Alibaba2		Patch		TESS	
	Anc.	Alt.	Anc.	Alt.	Anc.	Alt.
rs2428431 (SNP14)	MIG1, Sp1	None	Sp1, AML1	NF-E	TCF2 α , MIG1, AML1	NF-E, RAF
rs3801776 (SNP16)	None	1-Oct	None	None	None	RAF

^aOnly SNPs with p<0.01 in regulatory regions shown

“Because the *HOX* clusters are controlled by many different regulatory factors, SNPs in the intergenic regions of the clusters may be in enhancer or suppressor regions”. Therefore, each of the overtransmitted SNPs in these regions was evaluated using three transcription factor binding site prediction algorithms. SNP14 was predicted to change ancestral binding sites by all three programs, but the binding factors differed (Table 5.5). The ancestral allele of SNP16 was not identified as a potential binding site by any of the programs, while the alternative SNP generated a potential binding site by two of the programs (Table 5.5). SNP1 was not predicted to change any binding sites (data not shown).

SNPs within promoter regions have been shown to alter gene expression by affecting transcription factor binding sites¹⁵⁶. SNP16 is located in the basal promoter region of *HOXA9*. The sequence surrounding SNP16 was inputted into three different transcription factor binding site prediction programs, Alibaba2, Patch and Transcription Element Search Software (TESS)⁹³⁻⁹⁵. The results show a loss of a transcription factor binding site, although

the predicted site varies between programs. This suggests that the expression pattern of *HOXA9* may be affected by the presence of alternative alleles at SNP16.

Discussion

This study assessed the role of *HOX* genes in clubfoot. The T956A/M319K *HOXD10* mutation that has been shown to cause CVT in individuals with isolated clubfoot¹⁴⁸ was tested in the discovery population. This mutation was not found in cases or controls. This result confirms the previous finding of Dobbs et al. and provides further evidence that this mutation does not cause isolated clubfoot¹⁵⁷.

Next, we assessed variants in the *HOXA* and *HOXD* gene clusters for an association with clubfoot. SNP1, which is 3' of the *HOXD* cluster, had altered transmission but is not expected to affect *HOXD* genes expressed in the limbs^{31,33}. SNP14 and SNP16, both in the *HOXA* gene cluster, were significantly overtransmitted. "SNP14 is in intron 1 of *HOXA2* and changes predicted DNA binding sites [Table 5.5]. *HOXA2* is not expressed in the limb and is not likely to contribute to clubfoot, but we cannot exclude the possibility that these SNPs may regulate expression levels of other upstream 5' *HOXD* genes". SNP16, is located in the *HOXA9* basal promoter region, and showed altered transmission in nonHispanic whites in both the discovery and validation groups. Pairwise haplotypes involving SNP16 were overtransmitted and this SNP had gene-gene interactions. "*HOXA9* is expressed in both the proximal and distal limb and the variant allele is predicted to introduce novel DNA binding sites [Table 5.5]". This change may alter *HOXA9* expression levels and affect limb or muscle development.

Perturbations in limb developmental processes may affect the final positioning of the foot, as would abnormal muscle or tendon development that is associated with clubfoot

^{1,7,11,14}. We found that genetic variants in *HOXD* and *HOXA* are associated with clubfoot, and these variants may perturb gene expression and contribute to clubfoot. Additionally, we found variation in apoptotic genes interacts with variants in *HOXD* and *HOXA* genes, and these interactions are associated with clubfoot¹⁰⁶. These results are supported by work indicating that apoptosis is important for muscle morphogenesis²².

Apoptosis is associated with interdigital removal of excess skin; however, it has more recently been shown to play a far more subtle role in later limb development shaping muscle and tendons. Specifically, *Casp3* has been shown to modulate apoptosis in later muscle and tendon development. While we did not find a strong association with *Casp3* and clubfoot alone, we did find evidence for interactions between *Casp3* and variants in *HOX* genes. This implies that individual variation in *Casp3* may not be sufficient to cause clubfoot but that the interaction with genes in other limb and muscle developmental pathways, such as *HOXA* and *HOXD*, are necessary in order to disrupt the developmental process. (Ester, 2009)

The interactions seen between the mitochondrial mediated programmed cell death genes and *HOXA* and *HOXD* genes suggest that there may be a common pathway or regulatory element controlling these two pathways. Perturbations of this common regulatory pathway may also lead to clubfoot. Apoptosis in hind limb myocytes helps shape developing muscles and coordinates muscle-tendon connections²⁸. This process is regulated by retinoic acid, which has also been shown to control *HOX* genes^{22,158}.

The goal of these studies was to identify high-risk haplotypes that would contribute to clubfoot. The first step in this process is to identify genetic variants that are associated with clubfoot and to determine whether they interact. In this study we found evidence for an association between a basal promoter SNP in *HOXA9* and clubfoot, and provide further evidence that mitochondrial mediated apoptotic genes play a role in clubfoot. (Ester, 2009)

Chapter 6. Conclusions and Future Studies

Conclusions

Clubfoot is a common birth defect occurring in more than 135,000 live births worldwide each year^{39,159,160}. Treatment modalities such as casting and surgery have improved the long term outcome but residual foot and leg abnormalities often persist^{42,82,83}. Little is known about how clubfoot develops, and knowledge gained by studying abnormalities of foot development, such as clubfoot, may help identify the genes that are involved with the final stages of limb development and rotation. The information can be translated into improved genetic counseling. The overall goal of this project was to define genes that contribute to clubfoot to provide a better understanding about how this disorder develops.

These studies were undertaken to identify genetic variation that contributes to clubfoot. A previous study interrogated a clubfoot chromosomal deletion region and found that apoptotic genes, *Casp8* and *Casp10*, were associated with clubfoot⁷⁸. These studies follow from this previous work and had three objectives: 1) to determine if genes which act downstream of *Casp8* and *Casp10* in the mitochondrial mediated pathway are associated with clubfoot, 2) to identify genes involved in the apoptotic pathway within clubfoot deletion regions and test for association with clubfoot, and 3) to interrogate other candidate genes in the clubfoot deletion regions such as those involved in patterning of the limbs.

Candidate genes were selected from processes known to participate in limb development, particularly in muscle development, including apoptotic and HOX genes. Many of these candidate genes were located in chromosomal deletion regions associated with clubfoot⁶⁹. One SNP from each mitochondrial mediated apoptotic gene was moderately associated with clubfoot¹⁰⁶. This confirmed the previous study in which *Casp8* and *Casp10* were associated with clubfoot.

Because the role of a subset of apoptotic genes in clubfoot development was confirmed, genotyping was expanded to all genes in the clubfoot deletion regions known to be involved in the apoptotic pathway. Apoptotic genes were excellent candidate genes because they were the only class of genes found in all six clubfoot deletion regions. 192 SNPs across 29 genes were assessed for association with clubfoot. Of note, there was no overlap in significant SNPs in both NHW and Hispanic samples, although several gene family members were positive in each ethnicity. For the NHW sample, *TNIP2* was the most significant gene and *NFAT2* was most significant in Hispanics. Haplotype analysis confirmed these results. Gene-gene interactions were detected, and although the SNPs from the single SNP analysis weren't involved in these interactions, some of the same genes were involved. These results suggest that there is heterogeneity between individual clubfoot families, and when the same genes are involved in the disease process, there is also allelic heterogeneity between the genetic variants.

There are other candidate genes within the clubfoot deletion regions in addition to the apoptotic genes that were interrogated, including the *HOXD* cluster, which is a family of transcription factors involved in patterning of the limbs. *HOXA* and *HOXD* have redundant function in the limbs, and so variants in the *HOXA* and *HOXD* gene clusters were tested for an association with clubfoot. SNP14 and SNP16, both in the *HOXA* gene cluster, were both significantly overtransmitted. “SNP14 is in intron 1 of *HOXA2* and changes predicted DNA binding sites. SNP16, is located in the *HOXA9* basal promoter region, and showed altered transmission in nonHispanic whites in both the discovery and validation groups”. This change may alter *HOXA9* expression levels and possibly could perturb limb or muscle development.

Taken together, these results support our working model for clubfoot (Fig. 4.2). Individual variants in candidate genes may be necessary but not sufficient until combined with other clubfoot predisposing variants. Clubfoot is a malformation of the foot, and although the variants in these genes are found ubiquitously in all cell type, perturbation of gene expression may be limited to particular regions of the body, such as the lower limb. For example, a slight perturbation of expression in an apoptotic gene may have no global phenotype but when combined with another variant that is only be expressed in the limb, such as a *HOXD* polymorphism, clubfoot may result.

The information from these studies may be used to improve risk assessment and develop population-based genetic screening programs for clubfoot. Additionally, this information may translate into improved genetic counseling for clubfoot families. Determination of the genetic variation that contributes to clubfoot may allow for advances in detection and treatment methods that will diminish the medical and psychosocial impact that clubfoot has on the child and their families.

Future Studies

The mitochondrial mediated apoptotic gene SNPs were genotyped on a smaller sample set than the *HOX* and clubfoot deletion region apoptotic genes because of the continuing collection of samples from new clubfoot families. Currently these SNPs are being evaluated on the expanded sample set. Once completed, gene-gene interactions will be run between the mitochondrial mediated and the clubfoot deletion region apoptotic genes.

The SNPlex assay for the clubfoot deletion region apoptotic genes produced low call rates, and so another assay with higher fidelity, such as TaqMan, will need to be used to

validate the results presented in Chapter 4. In addition, the most positive results will need to be assessed in our case-control validation population.

Because maternal smoking has consistently been associated with clubfoot, smoking metabolism genes are currently being interrogated and significant findings will be submitted for gene-gene interaction analysis. The outcome may provide information about the role of smoking metabolism and apoptosis genes together in clubfoot.

References

- 1 Loren G, J, Karpinski N, C, Mubarak S, J: Clinical implications of clubfoot histopathology. *J Pediatr Orthop* 1998; **18**: 765-769.
- 2 Bohm M: Embryologic origin of clubfoot. *Journal of Bone & Joint Surgery* 1929; **11**: 229-259.
- 3 Irani RN, ; Sherman MS: The Patological Anatomy of Club Foot. *The Journal of Bone and Joint Surgery* 1963; **45**: 45-52.
- 4 Isaacs H, Handelsman J, E, Badenhorst M, Pickering A: The muscles in club foot--a histological histochemical and electron microscopic study. *J Bone Joint Surg Br* 1977; **59-B**: 465-472.
- 5 Zierath J, R, Hawley J, A: Skeletal muscle fiber type: influence on contractile and metabolic properties. *PLoS Biol* 2004; **2**: e348.
- 6 Berne RL, MN: Principles of Physiology. St. Louis, Mosyb Inc., 2000.
- 7 Fukuhara K, Schollmeier G, Uthoff H, K: The pathogenesis of club foot. A histomorphometric and immunohistochemical study of fetuses. *J Bone Joint Surg Br* 1994; **76**: 450-457.
- 8 Germiller J, A, Lerner A, L, Pacifico R, J, Loder R, T, Hensinger R, N: Muscle and tendon size relationships in a paralyzed chick embryo model of clubfoot. *Journal of Pediatric Orthopedics* 1998; **18**: 314-318.
- 9 Omeroglu S, Peker T, Omeroglu H, Gulekon N, Mungan T, Danisman N: Intrauterine structure of foot muscles in talipes equinovarus due to high-level myelomeningocele:

- a light microscopic study in fetal cadavers. *Journal of Pediatric Orthopaedics, Part B* 2004; **13**: 263-267.
- 10 Herceg M, B, Weiner D, S, Agamanolis D, P, Hawk D: Histologic and histochemical analysis of muscle specimens in idiopathic talipes equinovarus. *J Pediatr Orthop* 2006; **26**: 91-93.
- 11 Windisch G, Anderhuber F, Haldi-Brandle V, Exner G, U: Additional muscle in idiopathic club foot. *Eur J Pediatr Surg* 2006; **16**: 294-296.
- 12 Gupta P, Singla R, Gupta R, Jindal R, Bahadur R: Accessory soleus muscle in clubfoot deformity: a report in four feet. *J Pediatr Orthop B* 2007; **16**: 106-109.
- 13 Karapinar L, Kaya A, Altay T, Ozturk H, Surenkok F: Congenital clubfoot associated with an accessory soleus muscle. *J Am Podiatr Med Assoc* 2008; **98**: 408-413.
- 14 Dobbs M, B, Walton T, Gordon J, E, Schoenecker P, L, Gurnett C, A: Flexor digitorum accessorius longus muscle is associated with familial idiopathic clubfoot. *J Pediatr Orthop* 2005; **25**: 357-359.
- 15 Kubota H, Noguchi Y, Urabe K, Itokawa T, Nakashima Y, Iwamoto Y: Flexor digitorum longus accessorius in the club foot of an infant with Nager syndrome. *Arch Orthop Trauma Surg* 2001; **121**: 95-96.
- 16 Porter R, W: An anomalous muscle in children with congenital talipes. *Clin Anat* 1996; **9**: 25-27.
- 17 Siapkara A, Duncan R: Congenital talipes equinovarus: a review of current management. *J Bone Joint Surg Br* 2007; **89**: 995-1000.
- 18 Hulme A: The management of congenital talipes equinovarus. *Early Hum Dev* 2005; **81**: 797-802.

- 19 Alvarez C, , Tredwell S, , Keenan S, , Beauchamp R, , De Vera M, , Choit R, , Sawatzky B: Treatment of Idiopathic Clubfoot Utilizing Botulinum A Toxin: A New Method and Its Short Term Outcomes. *J Pediatr Ortho* 2004.
- 20 Delgado M, R, Wilson H, Johnston C, Richards S, Karol L: A preliminary report of the use of botulinum toxin type A in infants with clubfoot: four case studies. *J Pediatr Orthop* 2000; **20**: 533-538.
- 21 Kardon G: Muscle and tendon morphogenesis in the avian hind limb. *Development* 1998; **125**: 4019-4032.
- 22 Rodriguez-Guzman M, Montero J, A, Santesteban E, Ganan Y, Macias D, Hurle J, M: Tendon-muscle crosstalk controls muscle bellies morphogenesis, which is mediated by cell death and retinoic acid signaling. *Dev Biol* 2007; **302**: 267-280.
- 23 O'Rahilly R, Mèuller F: Human embryology & teratology. New York, Wiley-Liss, 2001.
- 24 Larsen WJ: Human embryology. New York, Churchill Livingstone, 1997.
- 25 Yang Y: Growth and patterning in the limb: signaling gradients make the decision. *Sci Signal* 2009; **2**: pe3.
- 26 Fernandez-Teran M, Ros M, A: The Apical Ectodermal Ridge: morphological aspects and signaling pathways. *Int J Dev Biol* 2008; **52**: 857-871.
- 27 Lee G, S, Kochhar D, M, Collins M, D: Retinoid-induced limb malformations. *Curr Pharm Des* 2004; **10**: 2657-2699.
- 28 Lewandoski M, Mackem S: Limb development: the rise and fall of retinoic acid. *Curr Biol* 2009; **19**: R558-561.

- 29 Motoyama J: Essential roles of Gli3 and sonic hedgehog in pattern formation and developmental anomalies caused by their dysfunction. *Congenit Anom (Kyoto)* 2006; **46**: 123-128.
- 30 Robert B, Lallemand Y: Anteroposterior patterning in the limb and digit specification: contribution of mouse genetics. *Dev Dyn* 2006; **235**: 2337-2352.
- 31 McGinnis W, Krumlauf R: Homeobox genes and axial patterning. *Cell* 1992; **68**: 283-302.
- 32 Mark M, Rijli FM, Chambon P: Homeobox genes in embryogenesis and pathogenesis. *Pediatr Res* 1997; **42**: 421-429.
- 33 Mark M, Rijli F, M, Chambon P: Homeobox genes in embryogenesis and pathogenesis. *Pediatr Res* 1997; **42**: 421-429.
- 34 Houghton L, Rosenthal N: Regulation of a muscle-specific transgene by persistent expression of Hox genes in postnatal murine limb muscle. *Dev Dyn* 1999; **216**: 385-397.
- 35 Zakany J, Duboule D: The role of Hox genes during vertebrate limb development. *Curr Opin Genet Dev* 2007; **17**: 359-366.
- 36 Morriss-Kay GM, Sokolova N: Embryonic development and pattern formation. *Faseb J* 1996; **10**: 961-968.
- 37 Zuzarte-Luis V, Hurler J, M: Programmed cell death in the embryonic vertebrate limb. *Semin Cell Dev Biol* 2005; **16**: 261-269.
- 38 Lochmiller C, Johnston D, Scott A, Risman M, Hecht J, T: Genetic epidemiology study of idiopathic talipes equinovarus. *Am J Med Genet* 1998; **79**: 90-96.

- 39 Beals RK: Club foot in the Maori: a genetic study of 50 kindreds. *N Z Med J* 1978; **88**: 144-146.
- 40 Chung C, S, Nemecek R, W, Larsen I, J, Ching G, H: Genetic and epidemiological studies of clubfoot in Hawaii. General and medical considerations. *Hum Hered* 1969; **19**: 321-342.
- 41 Moorthi RN, Hashmi SS, Langois P, Canfield M, Waller DK, Hecht JT: Idiopathic talipes equinovarus (ITEV) (clubfeet) in Texas. *Am J Med Genet A* 2005; **132**: 376-380.
- 42 Dietz F: The genetics of idiopathic clubfoot. *Clin Orthop Relat Res* 2002: 39-48.
- 43 Yang H, Y, Chung C, S, Nemecek R, W: A genetic analysis of clubfoot in Hawaii. *Genet Epidemiol* 1987; **4**: 299-306.
- 44 Wang J, H, Palmer R, M, Chung C, S: The role of major gene in clubfoot. *Am J Hum Genet* 1988; **42**: 772-776.
- 45 Morton N, E, MacLean C, J: Analysis of family resemblance. 3. Complex segregation of quantitative traits. *Am J Hum Genet* 1974; **26**: 489-503.
- 46 Rebbeck T, R, Dietz F, R, Murray J, C, Buetow K, H: A single-gene explanation for the probability of having idiopathic talipes equinovarus. *Am J Hum Genet* 1993; **53**: 1051-1063.
- 47 Kruse L, M, Dobbs M, B, Gurnett C, A: Polygenic threshold model with sex dimorphism in clubfoot inheritance: the Carter effect. *J Bone Joint Surg Am* 2008; **90**: 2688-2694.
- 48 Wang JH, Palmer RM, Chung CS: The role of major gene in clubfoot. *Am J Hum Genet* 1988; **42**: 772-776.

- 49 Morton NE, MacLean CJ: Analysis of family resemblance. 3. Complex segregation of quantitative traits. *Am J Hum Genet* 1974; **26**: 489-503.
- 50 de Andrade M, Barnholtz JS, Amos CI, Lochmiller C, Scott A, Risman M, Hecht JT: Segregation analysis of idiopathic talipes equinovarus in a Texan population. *Am J Med Genet* 1998; **79**: 97-102.
- 51 Yang HY, Chung CS, Nemechek RW: A genetic analysis of clubfoot in Hawaii. *Genet Epidemiol* 1987; **4**: 299-306.
- 52 Ching GH, Chung CS, Nemechek RW: Genetic and epidemiological studies of clubfoot in Hawaii: ascertainment and incidence. *Am J Hum Genet* 1969; **21**: 566-580.
- 53 Pryor G, A, Villar R, N, Ronen A, Scott P, M: Seasonal variation in the incidence of congenital talipes equinovarus. *J Bone Joint Surg Br* 1991; **73**: 632-634.
- 54 Barker S, L, Macnicol M, F: Seasonal distribution of idiopathic congenital talipes equinovarus in Scotland. *J Pediatr Orthop B* 2002; **11**: 129-133.
- 55 Wallander H, Hovelius L, Michaelsson K: Incidence of congenital clubfoot in Sweden. *Acta Orthop* 2006; **77**: 847-852.
- 56 Loder R, T, Drvaric D, M, Carney B, Hamby Z, Barker S, Chesney D, Maffulli N: Lack of seasonal variation in idiopathic talipes equinovarus. *J Bone Joint Surg Am* 2006; **88**: 496-502.
- 57 Dickinson K, C, Meyer R, E, Kotch J: Maternal smoking and the risk for clubfoot in infants. *Birth Defects Res A Clin Mol Teratol* 2008; **82**: 86-91.

- 58 Skelly A, C, Holt V, L, Mosca V, S, Alderman B, W: Talipes equinovarus and maternal smoking: a population-based case-control study in Washington state. *Teratology* 2002; **66**: 91-100.
- 59 Honein M, A, Paulozzi L, J, Moore C, A: Family history, maternal smoking, and clubfoot: an indication of a gene-environment interaction. *Am J Epidemiol* 2000; **152**: 658-665.
- 60 Strach EH: Club-foot through the centuries. *Prog Pediatr Surg* 1986; **20**: 215-237.
- 61 Engell V, Damborg F, Andersen M, Kyvik K, O, Thomsen K: Club foot: a twin study. *J Bone Joint Surg Br* 2006; **88**: 374-376.
- 62 Idelberger K, : Die Ergebnisse der Zwillingsforschung beim angeborenen Klumpfuß. *Verh Dtsch Orthop Ges* 1939; **33**: 272-276.
- 63 Barker S, Chesney D, Miedzybrodzka Z, Maffulli N: Genetics and epidemiology of idiopathic congenital talipes equinovarus. *J Pediatr Orthop* 2003; **23**: 265-272.
- 64 Wynne-Davies R: Family studies and the cause of congenital clubfoot. *J Bone Joint Surg Am* 1964; **46**: 445-452.
- 65 Moorthi R, N, Hashmi S, S, Langois P, Canfield M, Waller D, K, Hecht J, T: Idiopathic talipes equinovarus (ITEV) (clubfeet) in Texas. *Am J Med Genet A* 2005; **132**: 376-380.
- 66 Palmer R, M, Conneally P, M, Yu P, L: Studies of the inheritance of idiopathic talipes equinovarus. *Orthop Clin North Am* 1974; **5**: 99-108.
- 67 de Andrade M, Barnholtz J, S, Amos C, I, Lochmiller C, Scott A, Risman M, Hecht J, T: Segregation analysis of idiopathic talipes equinovarus in a Texan population. *Am J Med Genet* 1998; **79**: 97-102.

- 68 Collins A: Approaches to the identification of susceptibility genes. *Parasite Immunol* 2009; **31**: 225-233.
- 69 Brewer C, Holloway S, Zawalnyski P, Schinzel A, FitzPatrick D: A chromosomal deletion map of human malformations. *Am J Hum Genet* 1998; **63**: 1153-1159.
- 70 Brewer C, Holloway S, Zawalnyski P, Schinzel A, FitzPatrick D: A chromosomal duplication map of malformations: regions of suspected haplo- and triplolethality--and tolerance of segmental aneuploidy--in humans. *Am J Hum Genet* 1999; **64**: 1702-1708.
- 71 Dietz F, R, Cole W, G, Tosi L, L, Carroll N, C, Werner R, D, Comstock D, Murray J, C: A search for the gene(s) predisposing to idiopathic clubfoot. *Clin Genet* 2005; **67**: 361-362.
- 72 Shyy W, Dietz F, Dobbs M, B, Sheffield V, C, Morcuende J, A: Evaluation of CAND2 and WNT7a as candidate genes for congenital idiopathic clubfoot. *Clin Orthop Relat Res* 2009; **467**: 1201-1205.
- 73 Pinorini-Godly M, T, Myers S, R: HPLC and GC/MS determination of 4-aminobiphenyl haemoglobin adducts in fetuses exposed to the tobacco smoke carcinogen in utero. *Toxicology* 1996; **107**: 209-217.
- 74 Brockton N, Little J, Sharp L, Cotton S, C: N-acetyltransferase polymorphisms and colorectal cancer: a HuGE review. *Am J Epidemiol* 2000; **151**: 846-861.
- 75 Hecht J, T, Ester A, Scott A, Wise C, A, Iovannisci D, M, Lammer E, J, Langlois P, H, Blanton S, H: NAT2 variation and idiopathic talipes equinovarus (clubfoot). *Am J Med Genet A* 2007; **143**: 2285-2291.

- 76 Gurnett C, A, Alaei F, Desruisseau D, Boehm S, Dobbs M, B: Skeletal muscle contractile gene (TNNT3, MYH3, TPM2) mutations not found in vertical talus or clubfoot. *Clin Orthop Relat Res* 2009; **467**: 1195-1200.
- 77 Gurnett C, A, Alaei F, Kruse L, M, Desruisseau D, M, Hecht J, T, Wise C, A, Bowcock A, M, Dobbs M, B: Asymmetric lower-limb malformations in individuals with homeobox PITX1 gene mutation. *Am J Hum Genet* 2008; **83**: 616-622.
- 78 Heck A, L, Bray M, S, Scott A, Blanton S, H, Hecht J, T: Variation in CASP10 gene is associated with idiopathic talipes equinovarus. *J Pediatr Orthop* 2005; **25**: 598-602.
- 79 Li Z, G, Ji H, Fu W, N, Zhao Y, Y, Jin C, L, Ji S, J, Sun K, L: [Proteomic analysis of the ankle joint bone, ankle joint tissue and spinal cord of clubfoot-like deformity in rat fetuses]. *Zhonghua Yi Xue Yi Chuan Xue Za Zhi* 2007; **24**: 52-58.
- 80 Guo L, Zhao Y, Y, Ji S, J, Sun K, L, Dong L, Y: Effect of the regulation of IGFs system components in retinoic-acid induced congenital clubfoot. *Acta Genetica Sinica* 2004; **31**: 766-770.
- 81 Delgado-Baeza E, Santos-Alvarez I, Martos-Rodriguez A: Retinoic acid-induced clubfoot-like deformity: pathoanatomy in rat fetuses. *J Pediatr Orthop B* 1999; **8**: 12-18.
- 82 Ponseti IV, Campos J: Observations on the pathogenesis and treatment of congenital clubfoot. *Clin Orthop* 1972; **84**: 50-60.
- 83 Rodgveller B: Talipes Equinovarus. *Clin Podiatry* 1984; **1**: 477-499.
- 84 Barrett J, C, Fry B, Maller J, Daly M, J: Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005; **21**: 263-265.

- 85 Abecasis G, R, , Cookson W, O: GOLD--graphical overview of linkage disequilibrium. *Bioinformatics* 2000; **16**: 182-183.
- 86 Abecasis G, R, Cherny S, S, Cookson W, O, Cardon L, R: Merlin--rapid analysis of dense genetic maps using sparse gene flow trees. *Nat Genet* 2002; **30**: 97-101.
- 87 Abecasis GR, Cookson W, O.: GOLD--graphical overview of linkage disequilibrium. *Bioinformatics* 2000; **16**: 182-183.
- 88 Martin ER, Monks SA, Warren LL, Kaplan NL: A test for linkage and association in general pedigrees: The pedigree disequilibrium test. *Am J Hum Genet* 2000; **67**: 146-154.
- 89 Horvath S, Xu X, Laird NM: The family based association test method: strategies for studying general genotype-phenotype associations. *Eur J Hum Genet* 2001; **9**: 301-306.
- 90 Horvath S, , Xu X, , Laird N, M: The family based association test method: strategies for studying general genotype-phenotype associations. *Eur J Hum Genet* 2001; **9**: 301-306.
- 91 Chung R, H, Hauser E, R, Martin E, R: The APL test: extension to general nuclear families and haplotypes and examination of its robustness. *Hum Hered* 2006; **61**: 189-199.
- 92 Martin E, R, Ritchie M, D, Hahn L, Kang S, Moore J, H: A novel method to identify gene-gene effects in nuclear families: the MDR-PDT. *Genet Epidemiol* 2006; **30**: 111-123.
- 93 Grabe N: AliBaba2: context specific identification of transcription factor binding sites. *In Silico Biol* 2002; **2**: S1-15.

- 94 Matys V, Kel-Margoulis O, V, Fricke E, Liebich I, Land S, Barre-Dirrie A, Reuter I, Chekmenev D, Krull M, Hornischer K, Voss N, Stegmaier P, Lewicki-Potapov B, Saxel H, Kel A, E, Wingender E: TRANSFAC and its module TRANSCompel: transcriptional gene regulation in eukaryotes. *Nucleic Acids Research* 2006; **34**: D108-110.
- 95 Schug J: Using TESS to Predict Transcription Factor Binding Sites in DNA Sequence; in: Baxevanis AD (ed): Current Protocols in Bioinformatics, John Wiley and Sons, Inc., 2003, pp 2.6.1-2.6.15.
- 96 Adams V, Gielen S, Hambrecht R, Schuler G: Apoptosis in skeletal muscle. *Front Biosci* 2001; **6**: D1-D11.
- 97 Kurokawa M, Kornbluth S: Caspases and kinases in a death grip. *Cell* 2009; **138**: 838-854.
- 98 Kiechle F, L, Zhang X: Apoptosis: biochemical aspects and clinical implications. *Clin Chim Acta* 2002; **326**: 27-45.
- 99 Bhardwaj A, Aggarwal B, B: Receptor-mediated choreography of life and death. *J Clin Immunol* 2003; **23**: 317-332.
- 100 Slee E, A, Harte M, T, Kluck R, M, Wolf B, B, Casiano C, A, Newmeyer D, D, Wang H, G, Reed J, C, Nicholson D, W, Alnemri E, S, Green D, R, Martin S, J: Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. *J Cell Biol* 1999; **144**: 281-292.
- 101 Billen L, P, Shamas-Din A, Andrews D, W: Bid: a Bax-like BH3 protein. *Oncogene* 2008; **27 Suppl 1**: S93-104.

- 102 Szegezdi E, Macdonald D, C, Ni Chonghaile T, Gupta S, Samali A: Bcl-2 family on guard at the ER. *Am J Physiol Cell Physiol* 2009; **296**: C941-953.
- 103 Wong W, W, Puthalakath H: Bcl-2 family proteins: the sentinels of the mitochondrial apoptosis pathway. *IUBMB Life* 2008; **60**: 390-397.
- 104 Bao Q, Shi Y: Apoptosome: a platform for the activation of initiator caspases. *Cell Death Differ* 2007; **14**: 56-65.
- 105 Cohen G, M: Caspases: the executioners of apoptosis. *Biochem J* 1997; **326 (Pt 1)**: 1-16.
- 106 Ester A, R, Tyerman G, Wise C, A, Blanton S, H, Hecht J, T: Apoptotic gene analysis in idiopathic talipes equinovarus (clubfoot). *Clin Orthop Relat Res* 2007; **462**: 32-37.
- 107 Heck AL, Bray MS, Scott A, Blanton SH, Hecht JT: Variation in CASP10 gene is associated with idiopathic talipes equinovarus. *J Pediatr Orthop* 2005; **25**: 598-602.
- 108 Beals R, K: Club foot in the Maori: a genetic study of 50 kindreds. *N Z Med J* 1978; **88**: 144-146.
- 109 Lehman W: The Clubfoot. Philadelphia, J B Lippincott, 1980, pp 1-6.
- 110 Ponseti I, V, Pirani S, Dietz F, Morcuende J, A, Mosca V, Herzenberg J, E, Weinstein S, Penny N, Steenbeek M: Clubfoot: Ponseti Management. Global-HELP Organization, 2003.
- 111 Yang J, J: Mixed lineage kinase ZAK utilizing MKK7 and not MKK4 to activate the c-Jun N-terminal kinase and playing a role in the cell arrest. *Biochem Biophys Res Commun* 2002; **297**: 105-110.
- 112 Pichler A, Melchior F: Ubiquitin-related modifier SUMO1 and nucleocytoplasmic transport. *Traffic* 2002; **3**: 381-387.

- 113 Lin D, Y, Huang Y, S, Jeng J, C, Kuo H, Y, Chang C, C, Chao T, T, Ho C, C, Chen Y, C, Lin T, P, Fang H, I, Hung C, C, Suen C, S, Hwang M, J, Chang K, S, Maul G, G, Shih H, M: Role of SUMO-interacting motif in Daxx SUMO modification, subnuclear localization, and repression of sumoylated transcription factors. *Mol Cell* 2006; **24**: 341-354.
- 114 Huang T, T, Wuerzberger-Davis S, M, Wu Z, H, Miyamoto S: Sequential modification of NEMO/IKKgamma by SUMO-1 and ubiquitin mediates NF-kappaB activation by genotoxic stress. *Cell* 2003; **115**: 565-576.
- 115 Gu Q, Bowden G, T, Normolle D, Sun Y: SAG/ROC2 E3 ligase regulates skin carcinogenesis by stage-dependent targeting of c-Jun/AP1 and IkappaB-alpha/NF-kappaB. *J Cell Biol* 2007; **178**: 1009-1023.
- 116 Salaun B, Romero P, Lebecque S: Toll-like receptors' two-edged sword: when immunity meets apoptosis. *Eur J Immunol* 2007; **37**: 3311-3318.
- 117 Coux O, Goldberg A, L: Enzymes catalyzing ubiquitination and proteolytic processing of the p105 precursor of nuclear factor kappaB1. *J Biol Chem* 1998; **273**: 8820-8828.
- 118 Verstrepen L, Carpentier I, Verhelst K, Beyaert R: ABINs: A20 binding inhibitors of NF-kappa B and apoptosis signaling. *Biochem Pharmacol* 2009; **78**: 105-114.
- 119 Chuang TH, Ulevitch RJ: Triad3A, an E3 ubiquitin-protein ligase regulating Toll-like receptors. *Nat Immunol* 2004; **5**: 495-502.
- 120 Fearn C, Pan Q, Mathison J, C, Chuang T, H: Triad3A regulates ubiquitination and proteasomal degradation of RIP1 following disruption of Hsp90 binding. *J Biol Chem* 2006; **281**: 34592-34600.

- 121 Thome M, Weil R: Post-translational modifications regulate distinct functions of CARMA1 and BCL10. *Trends Immunol* 2007; **28**: 281-288.
- 122 Royuela M, Rodriguez-Berriguete G, Fraile B, Paniagua R: TNF-alpha/IL-1/NF-kappaB transduction pathway in human cancer prostate. *Histol Histopathol* 2008; **23**: 1279-1290.
- 123 Mori K, Ando K, Heymann D, Redini F: Receptor activator of nuclear factor-kappa B ligand (RANKL) stimulates bone-associated tumors through functional RANK expressed on bone-associated cancer cells? *Histol Histopathol* 2009; **24**: 235-242.
- 124 Luo S, Rubinsztein D, C: Huntingtin promotes cell survival by preventing Pak2 cleavage. *J Cell Sci* 2009; **122**: 875-885.
- 125 Calo V, Migliavacca M, Bazan V, Macaluso M, Buscemi M, Gebbia N, Russo A: STAT proteins: from normal control of cellular events to tumorigenesis. *J Cell Physiol* 2003; **197**: 157-168.
- 126 Yuan Z, Gong S, Luo J, Zheng Z, Song B, Ma S, Guo J, Hu C, Thiel G, Vinson C, Hu C, D, Wang Y, Li M: Opposing roles for ATF2 and c-Fos in c-Jun-mediated neuronal apoptosis. *Mol Cell Biol* 2009; **29**: 2431-2442.
- 127 Chai W, R, Wang Q, Gao H, B: NFAT2 is implicated in corticosterone-induced rat Leydig cell apoptosis. *Asian J Androl* 2007; **9**: 623-633.
- 128 Barlow C, A, Kitiphongspattana K, Siddiqui N, Roe M, W, Mossman B, T, Lounsbury K, M: Protein kinase A-mediated CREB phosphorylation is an oxidant-induced survival pathway in alveolar type II cells. *Apoptosis* 2008; **13**: 681-692.
- 129 Hitchens MR, Robbins PD: The role of the transcription factor DP in apoptosis. *Apoptosis* 2003; **8**: 461-468.

- 130 Hitchens M, R, Robbins P, D: The role of the transcription factor DP in apoptosis. *Apoptosis* 2003; **8**: 461-468.
- 131 Skapek S, X, Jansen D, Wei T, F, McDermott T, Huang W, Olson E, N, Lee E, Y: Cloning and characterization of a novel Kruppel-associated box family transcriptional repressor that interacts with the retinoblastoma gene product, RB. *J Biol Chem* 2000; **275**: 7212-7223.
- 132 Soni S, Bala S, Hanspal M: Requirement for erythroblast-macrophage protein (Emp) in definitive erythropoiesis. *Blood Cells Mol Dis* 2008; **41**: 141-147.
- 133 Park S, Y, Kang K, B, Thapa N, Kim S, Y, Lee S, J, Kim I, S: Requirement of adaptor protein GULP during stabilin-2-mediated cell corpse engulfment. *J Biol Chem* 2008; **283**: 10593-10600.
- 134 Shah S, Smith H, Feng X, Rancourt D, E, Riabowol K: ING function in apoptosis in diverse model systems. *Biochem Cell Biol* 2009; **87**: 117-125.
- 135 Myers K, Gagou M, E, Zuazua-Villar P, Rodriguez R, Meuth M: ATR and Chk1 suppress a caspase-3-dependent apoptotic response following DNA replication stress. *PLoS Genet* 2009; **5**: e1000324.
- 136 Ljungman M: The transcription stress response. *Cell Cycle* 2007; **6**: 2252-2257.
- 137 Larsen L, Ropke C: Suppressors of cytokine signalling: SOCS. *Apms* 2002; **110**: 833-844.
- 138 Tuson M, Garanto A, Gonzalez-Duarte R, Marfany G: Overexpression of CERKL, a gene responsible for retinitis pigmentosa in humans, protects cells from apoptosis induced by oxidative stress. *Mol Vis* 2009; **15**: 168-180.

- 139 Fischer M, D, Budak M, T, Bakay M, Gorospe J, R, Kjellgren D, Pedrosa-Domellof F, Hoffman E, P, Khurana T, S: Definition of the unique human extraocular muscle allotype by expression profiling. *Physiol Genomics* 2005; **22**: 283-291.
- 140 Hao H, Dong Y, B, Bowling M, T, Zhou H, S, McMasters K, M: Alteration of gene expression in melanoma cells following combined treatment with E2F-1 and doxorubicin. *Anticancer Res* 2006; **26**: 1947-1956.
- 141 Delhalle S, Blasius R, Dicato M, Diederich M: A Beginner's Guide to NF- κ B Signaling Pathways. *Ann N Y Acad Sci* 2004; **1030**: 1-13.
- 142 Martin E, R, Bass M, P, Gilbert J, R, Pericak-Vance M, A, Hauser E, R: Genotype-based association test for general pedigrees: the genotype-PDT. *Genet Epidemiol* 2003; **25**: 203-213.
- 143 Kerkhoff E, Ziff E, B: Deregulated messenger RNA expression during T cell apoptosis. *Nucleic Acids Res* 1995; **23**: 4857-4863.
- 144 Ester A, R, Weymouth K, S, Burt A, Wise C, A, Scott A, Gurnett C, A, Dobbs M, B, Blanton S, H, Hecht J, T: Altered transmission of HOX and apoptotic SNPs identify a potential common pathway for clubfoot. *Am J Med Genet A* 2009; **149A**: 2745-2752.
- 145 Carter C, O: The inheritance of common congenital malformations. *Prog Med Genet* 1965; **5**: 59-84.
- 146 Shaut CA, Keene DR, Sorensen LK, Li DY, Stadler HS: HOXA13 Is essential for placental vascular patterning and labyrinth endothelial specification. *PLoS Genet* 2008; **4**: e1000073.
- 147 Hall B, K, Miyake T: All for one and one for all: condensations and the initiation of skeletal development. *Bioessays* 2000; **22**: 138-147.

- 148 Dobbs M, B, Gurnett C, A, Pierce B, Exner G, U, Robarge J, Morcuende J, A, Cole W, G, Templeton P, A, Foster B, Bowcock A, M: HOXD10 M319K mutation in a family with isolated congenital vertical talus. *J Orthop Res* 2006; **24**: 448-453.
- 149 Shrimpton A, E, Levinsohn E, M, Yozawitz J, M, Packard D, S, J, Cady R, B, Middleton F, A, Persico A, M, Hootnick D, R: A HOX gene mutation in a family with isolated congenital vertical talus and Charcot-Marie-Tooth disease. *Am J Hum Genet* 2004; **75**: 92-96.
- 150 Carpenter E, M, Goddard J, M, Davis A, P, Nguyen T, P, Capecchi M, R: Targeted disruption of Hoxd-10 affects mouse hindlimb development. *Development* 1997; **124**: 4505-4514.
- 151 Tarchini B, Huynh T, H, Cox G, A, Duboule D: HoxD cluster scanning deletions identify multiple defects leading to paralysis in the mouse mutant Ironside. *Genes Dev* 2005; **19**: 2862-2876.
- 152 Akarsu A, N, Stoilov I, Yilmaz E, Sayli B, S, Sarfarazi M: Genomic structure of HOXD13 gene: a nine polyalanine duplication causes synpolydactyly in two unrelated families. *Hum Mol Genet* 1996; **5**: 945-952.
- 153 Muragaki Y, Mundlos S, Upton J, Olsen B, R: Altered growth and branching patterns in synpolydactyly caused by mutations in HOXD13. *Science* 1996; **272**: 548-551.
- 154 Stadler H, S, Higgins K, M, Capecchi M, R: Loss of Eph-receptor expression correlates with loss of cell adhesion and chondrogenic capacity in Hoxa13 mutant limbs. *Development* 2001; **128**: 4177-4188.
- 155 Rahimov F, Marazita M, L, Visel A, Cooper M, E, Hitchler M, J, Rubini M, Domann F, E, Govil M, Christensen K, Bille C, Melbye M, Jugessur A, Lie R, T, Wilcox A, J,

- Fitzpatrick D, R, Green E, D, Mossey P, A, Little J, Steegers-Theunissen R, P, Pennacchio L, A, Schutte B, C, Murray J, C: Disruption of an AP-2alpha binding site in an IRF6 enhancer is associated with cleft lip. *Nat Genet* 2008.
- 156 Yoshimura M, Kimura T, Ishii M, Ishii K, Matsuura T, Geshi E, Hosokawa M, Muramatsu M: Functional polymorphisms in carboxylesterase1A2 (CES1A2) gene involves specific protein 1 (Sp1) binding sites. *Biochem Biophys Res Commun* 2008; **369**: 939-942.
- 157 Gurnett CA, Keppel C, Bick J, Bowcock AM, Dobbs MB: Absence of HOXD10 Mutations in Idiopathic Clubfoot and Sporadic Vertical Talus. *Clin Orthop Relat Res* 2007.
- 158 Marshall H, Morrison A, Studer M, Popperl H, Krumlauf R: Retinoids and Hox genes. *Faseb J* 1996; **10**: 969-978.
- 159 Lochmiller C, Johnston D, Scott A, Risman M, Hecht JT: Genetic epidemiology study of idiopathic talipes equinovarus. *Am J Med Genet* 1998; **79**: 90-96.
- 160 Chung CS, Nemecek RW, Larsen IJ, Ching GH: Genetic and epidemiological studies of clubfoot in Hawaii. General and medical considerations. *Hum Hered* 1969; **19**: 321-342.

Vita

Audrey Ricaud Ester was born in Stanford, California to Becky and Gary Ester on May 26, 1982. Audrey graduated from Plano Senior High School in Plano, TX in May, 2000, and began classes at Texas A&M Universtiy that fall. She received her BS is Genetics in May 2004. Audrey attended the University of Texas Health Science Center Houston and was very involved in student government at the institutional and system-wide level, and also chaired the UTserve volunteer initiative for two years. Audrey was also a trainee on two separate training grants, and won several presentation awards. Audrey Ester received her PhD in the spring of 2010 and wishes to pursue a career in clinical coordination and regulatory affairs.

***Appendix A: Mitochondrial mediated apoptosis
supplemental tables.***

Table A1. LD plot for *CASP8* and *CASP10*

	rs3900115	rs3731714	rs3769825
rs3900115		0.835	0.891
rs3731714	0.919		0.905
rs3769825	0.926	1	

Significant LD values shown in yellow, NHW shown above the diagonal and Hispanics below the diagonal.

Table A2. LD plot for *CASP3*

	rs1049253	rs1049216	rs1049210	rs1405944	rs2696057	rs2720378	rs4647602	rs1405937
rs1049253		0.907	1	1	0.827	0.388	1	0.829
rs1049216	1		0.99	1	1	0.286	0.922	0.676
rs1049210	1	0.99		0	1	1	1	0.997
rs1405944	1	1	0		1	0.842	1	0.726
rs2696057	1	1	1	1		0.679	0.868	0.918
rs2720378	0.617	0.286	1	0.842	0.679		0.969	0.773
rs4647602	1	0.922	1	1	0.868	0.969		0.981
rs1405937	0.927	0.676	0.997	0.726	0.918	0.773	0.981	

Significant LD values shown in yellow, NHW shown above the diagonal and Hispanics below the diagonal.

Table A3. LD plot for *APAF-1*

	rs7310804	rs2278361	rs2288729	rs6538879	rs3782558	rs1866477	rs7968661
rs7310804		1	1	1	0.983	0.903	0.903
rs2278361	1		0.896	1	1	0.838	0.933
rs2288729	0.948	0.98		0.92	1	1	0.833
rs6538879	0.92	0.814	0.957		1	0.946	0.894
rs3782558	0.91	0.976	1	0.949		0.835	0.892
rs1866477	0.575	0.998	1	0.445	0.354		0.999
rs7968661	0.788	0.935	0.85	0.931	0.819	1	

Significant LD values shown in yellow, NHW shown above the diagonal and Hispanics below the diagonal.

Table A4. LD plot for *BCL-2*

	rs1564483	rs8083946	rs1801018	rs2551402	rs1809319
rs1564483		0.263	0.004	0.046	0.336
rs8083946	0.5		0.045	0.019	0.04
rs1801018	0.283	0.036		0.957	0.97
rs2551402	0.114	0.039	0.966		0.423
rs1809319	0.493	0.023	0.946	0.127	

Significant LD values shown in yellow, NHW shown above the diagonal and Hispanics below the diagonal.

Table A5. LD plot for *BID*

	rs8919	rs181399	rs2072392	rs8190315	rs181405	rs181410	rs5747351	rs3788284
rs8919		0.817	1	1	0.632	0.545	0.16	0.153
rs181399	0.946		0.999	0.999	0.927	0.704	0.566	0.519
rs2072392	0.774	0.973		0.705	0.999	1	0.205	0.66
rs8190315	0.229	0.996	0.697		0.145	1	1	1
rs181405	0.519	1	0.24	0.421		0.782	0.161	0.095
rsS181410	0.826	0.532	0.016	1	0.794		0.138	0.008
rs5747351	0.177	0.186	0.074	0.584	0.046	0.315		0.953
rs3788284	0.151	0.07	0.334	0.489	0.049	0.261	0.881	

Significant LD values shown in yellow, NHW shown above the diagonal and Hispanics below the diagonal.

***Appendix B: Clubfoot Chromosomal Deletion Region
Apoptotic Genes Supplemental Tables***

Table B1. SNP locations, call rates and minor allele frequency (MAF)

Gene	Chr	SNP	BP Pos.	Location	Allele 1	Allele 2	Call Rate	NHW MAF	Hisp. CAF
ZAK	2	rs989531	173641864	Upstream	C	T	68.4	0.34	0.34
ZAK	2	rs6433395	173652126	Intron 1	C	T	75.0	0.48	0.33
ZAK	2	rs6759787	173668095	Intron 2	C	T	72.2	0.40	0.44
ZAK	2	rs17302977	173681546	Intron 2	C	T	66.9	0.43	0.20
ZAK	2	rs3769192	173700047	Intron 2	C	T	69.1	0.44	0.28
ZAK	2	rs4344898	173718318	Intron 2	G	T	74.8	0.41	0.42
ZAK	2	rs13032010	173741939	Intron 2	A	G	70.6	0.38	0.22
ZAK	2	rs1837470	173756617	Intron 4	C	T	72.3	0.48	0.59
ZAK	2	rs4972533	173779131	Intron 8	G	T	68.8	0.39	0.24
ZAK	2	rs2028382	173795993	Intron					
ZAK	2	rs2028382	173795993	10/3'UTR	G	T	70.6	0.49	0.33
ZAK	2	rs11685001	173812993	Intron 14	A	G	66.3	0.48	0.33
ZAK	2	rs11686011	173826292	Intron 14	A	G	66.4	0.43	0.23
ZAK	2	rs12618933	173844602	Downstream	C	T	74.1	0.49	0.28
CREB2	2	rs212352	175651527	Intron 12	C	T	73.7	0.33	0.40
CREB2	2	rs2698545	175697794	Intron 4	A	G	73.3	0.16	0.20
CREB2	2	rs10930693	175708457	Intron 3	C	T	71.1	0.23	0.28
CERKL	2	rs1047307	182109997	3' UTR	A	G	74.9	0.39	0.43
CERKL	2	rs11680383	182127394	Intron 6	C	T	66.5	0.35	0.24
CERKL	2	rs895901	182145339	Intron 3	A	T	74.8	0.28	0.33
CERKL	2	rs10445770	182168959	Intron 2	A	C	68.5	0.45	0.38
CERKL	2	rs1992394	182186910	Intron 1	C	T	70.3	0.45	0.43
CERKL	2	rs935087	182212013	Intron 1	A	G	64.5	0.37	0.34
CERKL	2	rs1866888	182235205	Upstream	C	T	72.7	0.45	0.40
GULP1	2	rs10931346	188862356	Upstream	C	T	71.1	0.44	0.60
GULP1	2	rs7593546	188877480	Intron 1	A	G	73.0	0.41	0.28
GULP1	2	rs4396679	188898949	Intron 1	G	T	73.1	0.40	0.28
GULP1	2	rs7586390	188922938	Intron 1	A	G	73.3	0.49	0.34
GULP1	2	rs12624002	188969212	Intron 2	G	T	69.9	0.46	0.50
GULP1	2	rs12474692	188989874	Intron 2	A	G	71.3	0.44	0.32
GULP1	2	rs10931359	189016529	Intron 2	A	G	66.4	0.39	0.52
GULP1	2	rs6724428	189085754	Intron 4	A	G	72.7	0.50	0.60
GULP1	2	rs11685321	189105316	Intron 6	A	G	72.2	0.43	0.34
GULP1	2	rs13034731	189122609	Intron 7	C	T	70.3	0.36	0.28
GULP1	2	rs1354905	189154716	Intron 8	A	G	73.6	0.49	0.46
STAT1	2	rs12468579	191540509	Downstream	A	G	73.7	0.44	0.51
STAT1	2	rs13395505	191546759	Intron 24	A	G	67.7	0.46	0.37
STAT1	2	rs2280233	191558811	Intron 14	A	G	71.0	0.45	0.32
STAT1	2	rs7562024	191563766	Intron 11	C	T	70.3	0.34	0.48
STAT1	2	rs6751855	191593016	Upstream	A	G	43.3	0.37	0.41
STAT4	2	rs925847	191605785	Intron 21	C	T	72.4	0.32	0.50
STAT4	2	rs16833215	191622044	Intron 13	A	G	51.2	0.37	0.59
STAT4	2	rs1517352	191639709	Intron 6	A	C	83.1	0.36	0.28
STAT4	2	rs16833249	191656517	Intron 3	C	T	74.0	0.37	0.41
STAT4	2	rs11693480	191665940	Intron 3	A	C	72.4	0.43	0.47
STAT4	2	rs12463658	191673589	Intron 3	A	C	71.3	0.34	0.26

Gene	Chr	SNP	BP Pos.	Location	Allele 1	Allele 2	Call Rate	NHW MAF	Hisp. CAF
<i>STAT4</i>	2	rs4853543	191684879	Intron 3	A	G	71.6	0.50	0.72
<i>STAT4</i>	2	rs4341967	191692531	Intron 3	A	T	71.1	0.44	0.40
<i>STAT4</i>	2	rs6738544	191697601	Intron 3	A	C	68.9	0.37	0.39
<i>STAT4</i>	2	rs7574909	191706191	Intron 3	C	T	72.7	0.35	0.43
<i>STAT4</i>	2	rs2356350	191710783	Intron 3	A	G	67.1	0.39	0.36
<i>STAT4</i>	2	rs11685878	191717700	Intron 3	C	T	55.9	0.33	0.30
<i>STAT4</i>	2	rs7572482	191723317	Intron 1	A	G	70.7	0.46	0.35
<i>STAT4</i>	2	rs897200	191726016	Upstream	A	G	88.0	0.38	0.32
<i>SUMO1</i>	2	rs6717044	202778312	Downstream	C	T	78.5	0.40	0.25
<i>SUMO1</i>	2	rs4675272	202794974	Intron 1	C	G	83.5	0.40	0.22
<i>SUMO1</i>	2	rs6755690	202798838	Intron 1	C	G	81.8	0.48	0.35
<i>SUMO1</i>	2	rs6709162	202806804	Intron 1	C	T	83.7	0.50	0.61
<i>SUMO1</i>	2	rs3754931	202811646	Upstream	C	T	79.4	0.46	0.57
<i>CREB1</i>	2	rs2253206	208100223	Upstream	A	G	70.9	0.42	0.34
<i>CREB1</i>	2	rs2551640	208116138	Intron 1	A	G	72.4	0.45	0.46
<i>CREB1</i>	2	rs10932201	208134502	Intron 4	A	G	72.5	0.42	0.40
<i>CREB1</i>	2	rs2254137	208152273	Intron 8	A	C	71.5	0.40	0.44
<i>SPSB4</i>	3	rs1108693	142351405	Upstream	C	T	73.4	0.43	0.45
<i>RNF7</i>	3	rs1980191	142939365	Upstream	A	T	67.8	0.49	0.45
<i>RNF7</i>	3	rs6769676	142944537	Intron 1	G	T	69.9	0.33	0.22
<i>RNF7</i>	3	rs6776205	142955345	Downstream	C	G	73.4	0.44	0.55
<i>TFDP2</i>	3	rs2163294	143151143	Downstream	A	C	68.3	0.44	0.41
<i>TFDP2</i>	3	rs7642874	143180562	Intron 3	C	T	73.2	0.40	0.33
<i>TFDP2</i>	3	rs9877536	143200426	Intron 1	C	G	67.5	0.49	0.34
<i>TFDP2</i>	3	rs13065446	143220532	Upstream	C	T	63.7	0.40	0.29
<i>ATR</i>	3	rs9816736	143655470	Intron 43	C	T	68.2	0.43	0.53
<i>ATR</i>	3	rs3922730	143685547	Intron 34	A	T	68.2	0.44	0.52
<i>ATR</i>	3	rs4273389	143696620	Intron 31	A	G	66.9	0.49	0.39
<i>ATR</i>	3	rs6440085	143715433	Intron 24	A	T	68.3	0.46	0.51
<i>ATR</i>	3	rs7651071	143726540	Intron 21	A	T	70.0	0.50	0.42
<i>ATR</i>	3	rs13085998	143746502	Intron 16	C	T	66.0	0.43	0.51
<i>ATR</i>	3	rs2227928	143764302	Intron 4	C	T	62.4	0.43	0.28
<i>ATR</i>	3	rs6792259	143784129	Upstream	A	T	72.2	0.47	0.48
<i>MAEA</i>	4	rs1680073	1270337	Upstream	C	T	70.0	0.47	0.33
<i>MAEA</i>	4	rs11727167	1275521	Intron 1	A	G	72.0	0.45	0.53
<i>MAEA</i>	4	rs7673398	1290077	Intron 1	A	G	64.9	0.40	0.32
<i>MAEA</i>	4	rs12641735	1294434	Intron 1	C	G	73.7	0.23	0.16
<i>MAEA</i>	4	rs12642410	1298409	Intron 2	A	G	72.8	0.47	0.45
<i>MAEA</i>	4	rs1316393	1305619	Intron 3	A	G	73.1	0.40	0.31
<i>MAEA</i>	4	rs7664474	1319116	Intron 6	A	T	75.2	0.37	0.42
<i>MAEA</i>	4	rs12647145	1328618	Downstream	A	C	69.9	0.28	0.16
<i>TNIP2</i>	4	rs9683949	2714278	Intron 5	C	T	69.1	0.40	0.38
<i>TNIP2</i>	4	rs4690055	2718461	Intron 2	A	G	72.1	0.49	0.71
<i>TNIP2</i>	4	rs4690060	2730020	Upstream	A	G	68.2	0.43	0.39
<i>HTT</i>	4	rs762855	3044593	Upstream	C	T	65.4	0.37	0.39
<i>HTT</i>	4	rs2285086	3059057	Intron 2	C	T	60.8	0.49	0.42
<i>HTT</i>	4	rs10015979	3079240	Intron 6	A	G	71.6	0.44	0.36
<i>HTT</i>	4	rs6446723	3096611	Intron 10	C	T	68.3	0.42	0.48

Gene	Chr	SNP	BP Pos.	Location	Allele 1	Allele 2	Call Rate	NHW MAF	Hisp. CAF
<i>HTT</i>	4	rs6855981	3118074	Intron 19	A	G	70.8	0.40	0.36
<i>HTT</i>	4	rs4690074	3131854	Intron 23	C	T	66.0	0.44	0.33
<i>HTT</i>	4	rs363096	3149819	Intron 28	A	G	61.2	0.27	0.35
<i>HTT</i>	4	rs363092	3165827	Intron 34	G	T	65.0	0.39	0.36
<i>HTT</i>	4	rs362336	3183630	Intron 39	C	T	65.1	0.33	0.30
<i>HTT</i>	4	rs362331	3185633	Intron 41	C	T	64.0	0.46	0.35
<i>HTT</i>	4	rs2269478	3204626	Intron 51	A	C	64.4	0.38	0.32
<i>TLR10</i>	4	rs10776482	38451180	Exon 4 (synon)	C	T	69.8	0.40	0.25
<i>TLR10</i>	4	rs11096955	38452502	Exon 4 (I369L)	A	C	67.5	0.40	0.22
<i>TLR10</i>	4	rs11096957	38452886	Exon 4 (N241H)	A	C	70.2	0.48	0.35
<i>TLR10</i>	4	rs7658893	38458815	Intron 1	A	G	69.9	0.50	0.61
<i>TLR1</i>	4	rs4833095	38476105	Exon 4 (N248S)	C	T	67.9	0.46	0.57
<i>TLR1</i>	4	rs5743565	38482378	Intron 1	A	G	71.7	0.42	0.34
<i>TLR6</i>	4	rs5743818	38505558	Exon 1 (synon)	G	T	69.7	0.45	0.46
<i>TLR6</i>	4	rs3821985	38506407	Exon 1 (synon)	C	G	63.0	0.34	0.34
<i>TLR6</i>	4	rs5743810	38506745	Exon 1 (S249P)	C	T	70.7	0.42	0.40
<i>WDR19</i>	4	rs1451821	38856812	Upstream	A	C	62.5	0.40	0.44
<i>WDR19</i>	4	rs6815686	38876186	Intron 5	A	T	68.9	0.43	0.45
<i>WDR19</i>	4	rs9997015	38900692	Intron 12	A	G	71.9	0.49	0.45
<i>WDR19</i>	4	rs9998591	38918506	Intron 17	A	C	67.9	0.33	0.22
<i>WDR19</i>	4	rs11096987	38935585	Intron 22	A	G	71.1	0.44	0.55
<i>WDR19</i>	4	rs3733280	38947936	Intron 25	A	G	65.2	0.44	0.41
<i>WDR19</i>	4	rs12648082	38956119	Intron 29	C	T	68.4	0.40	0.33
<i>WDR19</i>	4	rs1057807	38965868	Downstream	C	T	58.5	0.49	0.34
<i>UBE2K</i>	4	rs3912392	39375133	Upstream	A	G	75.1	0.40	0.29
<i>UBE2K</i>	4	rs13122400	39390138	Intron 1	C	T	55.7	0.43	0.53
<i>UBE2K</i>	4	rs12644528	39398637	Intron 1	C	T	74.9	0.44	0.52
<i>UBE2K</i>	4	rs302947	39419957	Intron 2	C	T	72.3	0.49	0.39
<i>UBE2K</i>	4	rs305827	39436384	Intron 4	G	T	73.3	0.46	0.51
<i>UBE2K</i>	4	rs10440307	39442582	Intron 4	C	T	41.3	0.50	0.42
<i>UBE2K</i>	4	rs4263408	39461671	Downstream	C	T	75.9	0.43	0.51
<i>CARMA1</i>	7	rs11982651	2908799	Downstream	C	T	70.3	0.43	0.28
<i>CARMA1</i>	7	rs1713911	2928435	Intron 15	A	C	66.9	0.47	0.48
<i>CARMA1</i>	7	rs1476636	2950046	Intron 5	C	T	64.4	0.47	0.33
<i>CARMA1</i>	7	rs4722276	2985330	Intron 1	C	G	64.1	0.45	0.53
<i>CARMA1</i>	7	rs12538346	2997820	Intron 1	C	T	66.9	0.40	0.32
<i>CARMA1</i>	7	rs10236776	3015535	Intron 1	A	G	70.5	0.23	0.16
<i>CARMA1</i>	7	rs4722356	3044023	Intron 1	C	G	71.3	0.47	0.45
<i>RBAK</i>	7	rs7805748	5048563	Upstream	C	T	72.2	0.40	0.31
<i>RBAK</i>	7	rs10238244	5062844	Intron 2	C	T	70.4	0.37	0.42
<i>RBAK</i>	7	rs7778444	5074398	3' UTR	A	G	70.6	0.28	0.16
<i>TRIAD3</i>	7	rs852374	5642645	Downstream	A	G	72.2	0.40	0.38

Gene	Chr	SNP	BP Pos.	Location	Allele 1	Allele 2	Call Rate	NHW MAF	Hisp. CAF
<i>TRIAD3</i>	7	rs13246406	5647119	Intron 15	C	T	72.7	0.49	0.71
<i>TRIAD3</i>	7	rs852522	5660069	Intron 13	C	T	68.5	0.43	0.39
<i>TRIAD3</i>	7	rs13247447	5665490	Intron 13	A	G	69.3	0.37	0.39
<i>TRIAD3</i>	7	rs852417	5670195	Intron 13	C	T	13.9	0.49	0.42
<i>TRIAD3</i>	7	rs3823681	5675186	Intron 13	C	T	72.3	0.44	0.36
<i>TRIAD3</i>	7	rs3779092	5679126	Intron 13	C	T	71.8	0.42	0.48
<i>TRIAD3</i>	7	rs852394	5687974	Intron 13	C	T	82.2	0.40	0.36
<i>TRIAD3</i>	7	rs1468996	5700983	Intron 13	A	G	78.5	0.44	0.33
<i>TRIAD3</i>	7	rs2302907	5727386	Intron 8	C	T	80.4	0.27	0.35
<i>TRIAD3</i>	7	rs13239194	5736015	Intron 6	C	T	69.4	0.39	0.36
<i>TRIAD3</i>	7	rs13237614	5740628	Intron 5	G	T	78.7	0.33	0.30
<i>TRIAD3</i>	7	rs2112006	5752771	Intron 3	C	T	77.9	0.46	0.35
<i>TRIAD3</i>	7	rs11771172	5760746	Intron 2	C	T	79.6	0.38	0.32
<i>TRIAD3</i>	7	rs6971918	5768152	Intron 1	C	T	83.9	0.40	0.25
<i>TRIAD3</i>	7	rs10257204	5773667	Intron 1	A	G	80.5	0.40	0.22
<i>TRIAD3</i>	7	rs6967635	5780459	Intron 1	G	T	87.2	0.48	0.35
<i>TRIAD3</i>	7	rs2017620	5802244	Upstream	C	T	73.8	0.50	0.61
<i>TNFSF13B</i>	13	rs9514828	107719374	Upstream	C	T	85.4	0.46	0.57
<i>TNFSF13B</i>	13	rs8181791	107732046	Intron 1	A	G	62.2	0.42	0.34
<i>TNFSF13B</i>	13	rs10508198	107740789	Intron 2	C	G	60.2	0.45	0.46
<i>TNFSF13B</i>	13	rs9520835	107754318	Intron 3	A	G	84.0	0.34	0.34
<i>TNFSF13B</i>	13	rs1224163	107763060	Downstream	G	T	85.8	0.42	0.40
<i>ING1</i>	13	rs4773240	110158586	Upstream	A	G	67.0	0.40	0.44
<i>ING1</i>	13	rs1441043	110165651	Intron 1	A	G	63.0	0.43	0.45
<i>ING1</i>	13	rs6492308	110175830	Downstream	C	T	70.9	0.49	0.45
<i>TFDP1</i>	13	rs7325214	113284925	Upstream	C	T	67.7	0.33	0.22
<i>TFDP1</i>	13	rs4150703	113300578	Intron 2	A	G	61.8	0.44	0.55
<i>TFDP1</i>	13	rs9577595	113316202	Intron 3	C	T	71.5	0.44	0.41
<i>TFDP1</i>	13	rs12428926	113332373	Intron 4	C	T	71.5	0.04	0.33
<i>TFDP1</i>	13	rs4150832	113343955	Downstream	C	T	65.1	0.49	0.34
<i>TNFRSF11A</i>	18	rs2981007	58133882	Upstream	C	T	79.2	0.40	0.29
<i>TNFRSF11A</i>	18	rs4941125	58148664	Intron 1	A	G	82.7	0.43	0.53
<i>TNFRSF11A</i>	18	rs7239261	58156026	Intron 1	A	C	85.4	0.44	0.52
<i>TNFRSF11A</i>	18	rs4263037	58167213	Intron 2	A	G	82.7	0.49	0.39
<i>TNFRSF11A</i>	18	rs7236060	58174262	Intron 4	A	G	85.1	0.46	0.51
<i>TNFRSF11A</i>	18	rs8094884	58179108	Intron 6	A	G	77.2	0.50	0.42
<i>TNFRSF11A</i>	18	rs8089829	58182884	Intron 7	A	G	74.0	0.43	0.51
<i>TNFRSF11A</i>	18	rs12959396	58190289	Intron 9	G	T	82.0	0.43	0.28
<i>TNFRSF11A</i>	18	rs9646629	58202179	Intron 9	C	G	26.6	0.47	0.48
<i>TNFRSF11A</i>	18	rs2957125	58209322	Downstream	A	T	52.8	0.47	0.33
<i>SOCS6</i>	18	rs7230661	66099091	Upstream	A	G	83.7	0.45	0.53
<i>SOCS6</i>	18	rs713130	66111574	Intron 1	C	T	74.5	0.40	0.32
<i>SOCS6</i>	18	rs2053420	66118259	Intron 1	C	T	83.6	0.23	0.16
<i>NFAT2</i>	18	rs9962479	75256172	Upstream	A	G	64.3	0.47	0.45
<i>NFAT2</i>	18	rs8090692	75264917	Intron 2	A	G	82.5	0.40	0.31
<i>NFAT2</i>	18	rs2036892	75269524	Intron 2	A	T	84.9	0.37	0.42
<i>NFAT2</i>	18	rs4799055	75282991	Intron 3	G	T	79.6	0.28	0.16
<i>NFAT2</i>	18	rs8097537	75294234	Intron 3	C	T	82.8	0.40	0.38

Gene	Chr	SNP	BP Pos.	Location	Allele 1	Allele 2	Call Rate	NHW MAF	Hisp. CAF
<i>NFAT2</i>	18	rs12608349	75300626	Intron 4	C	T	80.4	0.49	0.71
<i>NFAT2</i>	18	rs2290154	75312163	Intron 6	C	T	78.1	0.43	0.39
<i>NFAT2</i>	18	rs7227107	75328464	Exon 9 (synon)	A	G	37.9	0.37	0.39
<i>NFAT2</i>	18	rs370989	75348674	Intron 10	C	G	85.5	0.49	0.42
<i>NFAT2</i>	18	rs1667673	75353032	Intron 10	C	G	79.0	0.44	0.36
<i>NFAT2</i>	18	rs1660139	75371686	Intron 10	A	G	83.3	0.42	0.48
<i>NFAT2</i>	18	rs177820	75377952	Intron 10	C	T	76.5	0.40	0.36
<i>NFAT2</i>	18	rs3894049	75385918	Intron 10	C	G	86.2	0.44	0.33
<i>NFAT2</i>	18	rs183374	75392406	Downstream	A	G	83.5	0.27	0.35

Chr=chromosome; BP Pos.=base pair position; NHW=nonHispanic white; MAF=minor allele frequency; Hisp.=Hispanic; CAF=corresponding allele frequency; synon=synonymous; blue squares denote SNPs out of Hardy-Weinberg Equilibrium.

Table B2. Association of apoptotic genes with clubfoot in nonHispanic whites.

Gene	Chr	SNP	NHW			NHW FHx			NHW Trios		
			APL	PDT	GenPDT	APL	PDT	GenPDT	APL	PDT	GenPDT
ZAK	2	rs989531	0.794	0.383	0.482	0.273	0.674	0.185	0.300	0.276	0.461
ZAK	2	rs6433395	0.039	0.591	0.064	0.241	0.842	0.647	0.068	0.109	0.011
ZAK	2	rs6759787	0.107	0.125	0.288	0.061	0.098	0.181	0.813	0.782	0.911
ZAK	2	rs17302977	0.616	0.712	0.947	0.843	0.652	0.929	0.785	1.000	0.924
ZAK	2	rs3769192	0.012	0.272	0.548	0.281	0.933	0.988	0.011	0.071	0.212
ZAK	2	rs4344898	0.393	0.867	0.879	0.751	0.409	0.666	0.428	0.387	0.629
ZAK	2	rs13032010	0.034	0.187	0.401	0.318	0.334	0.666	0.035	0.317	0.361
ZAK	2	rs1837470	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
ZAK	2	rs4972533	0.557	0.657	0.919	0.866	0.610	0.865	0.567	1.000	0.918
ZAK	2	rs2028382	0.519	0.649	0.707	0.799	0.466	0.565	0.247	0.668	0.903
ZAK	2	rs11685001	0.198	0.098	0.180	0.595	0.265	0.467	0.144	0.166	0.308
ZAK	2	rs11686011	0.314	0.543	0.663	0.104	0.363	0.138	0.668	0.655	0.447
ZAK	2	rs12618933	0.485	0.281	0.622	0.339	0.317	0.586	0.990	0.647	0.632
ATF2/CREB2	2	rs212352	0.043	0.154	0.212	0.263	0.490	0.378	0.089	0.061	0.134
ATF2/CREB2	2	rs2698545	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
ATF2/CREB2	2	rs10930693	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
CERKL	2	rs1047307	0.931	1.000	0.968	0.654	0.785	0.910	0.459	0.639	0.893
CERKL	2	rs11680383	0.345	N/A	N/A	0.608	N/A	N/A	0.430	N/A	N/A
CERKL	2	rs895901	0.303	0.312	0.548	0.405	0.224	0.188	0.568	0.893	0.334
CERKL	2	rs10445770	0.330	0.350	0.332	0.791	0.590	0.355	0.073	0.345	0.670
CERKL	2	rs1992394	0.050	0.147	0.279	0.354	0.248	0.293	0.071	0.376	0.318
CERKL	2	rs935087	0.493	0.466	0.728	0.434	0.541	0.817	0.913	0.691	0.525
CERKL	2	rs1866888	0.862	0.519	0.443	0.712	0.526	0.526	0.814	0.895	0.817
GULP1	2	rs10931346	0.249	0.302	0.613	0.856	0.155	0.248	0.127	0.674	0.297
GULP1	2	rs7593546	0.779	0.904	0.694	0.127	0.427	0.648	0.021	0.132	0.271
GULP1	2	rs4396679	0.095	0.826	0.447	0.803	0.735	0.302	0.016	0.307	0.622
GULP1	2	rs7586390	0.691	0.844	0.937	0.385	0.363	0.550	0.081	0.233	0.057
GULP1	2	rs12624002	0.746	0.773	0.876	0.512	0.746	0.954	0.811	1.000	0.696
GULP1	2	rs12474692	0.136	0.938	0.646	0.869	0.233	0.371	0.027	0.083	0.219
GULP1	2	rs10931359	0.247	0.212	0.307	0.507	0.927	0.792	0.006	0.025	0.112
GULP1	2	rs6724428	0.116	1.000	0.344	0.396	0.807	0.658	0.100	0.639	0.348
GULP1	2	rs11685321	0.110	0.140	0.352	0.329	0.351	0.571	0.201	0.180	0.243
GULP1	2	rs13034731	0.124	0.957	0.995	0.483	0.732	0.888	0.145	0.446	0.640
GULP1	2	rs1354905	0.734	0.175	0.460	0.400	0.665	0.879	0.208	0.047	0.160
STAT1	2	rs12468579	0.406	0.785	0.561	0.814	0.211	0.326	0.291	0.095	0.209
STAT1	2	rs13395505	0.325	0.292	0.521	0.977	0.270	0.482	0.127	0.847	0.953
STAT1	2	rs2280233	0.767	0.632	0.710	0.271	0.901	0.821	0.525	0.547	0.801
STAT1	2	rs7562024	0.508	0.895	0.973	0.216	0.882	0.969	0.724	1.000	1.000
STAT1	2	rs6751855	0.425	0.243	0.154	0.577	0.066	0.173	0.533	0.599	0.178
STAT4	2	rs925847	0.701	0.558	0.401	0.526	0.895	0.982	0.875	0.317	0.032
STAT4	2	rs16833215	0.580	0.635	0.851	0.913	0.782	0.241	0.514	0.647	0.098
STAT4	2	rs1517352	0.830	0.541	0.826	0.207	0.307	0.562	0.345	0.612	0.860
STAT4	2	rs16833249	0.465	0.567	0.216	0.079	0.836	0.092	0.329	0.052	0.180
STAT4	2	rs11693480	0.817	0.484	0.328	0.610	0.541	0.522	0.377	0.695	0.400
STAT4	2	rs12463658	0.894	0.498	0.692	0.482	0.363	0.696	0.292	0.763	0.574
STAT4	2	rs4853543	0.863	0.394	0.052	0.930	0.330	0.020	0.622	1.000	1.000

Gene	Chr	SNP	NHW			NHW FHx			NHW Trios		
			APL	PDT	GenPDT	APL	PDT	GenPDT	APL	PDT	GenPDT
<i>STAT4</i>	2	rs4341967	0.656	0.574	0.695	0.892	0.915	0.730	0.420	0.332	0.578
<i>STAT4</i>	2	rs6738544	0.506	0.878	0.071	0.270	0.602	0.053	0.833	0.194	0.429
<i>STAT4</i>	2	rs7574909	0.227	0.768	0.926	0.396	0.592	0.864	0.358	0.617	0.783
<i>STAT4</i>	2	rs2356350	0.976	0.118	0.218	0.385	0.439	0.764	0.333	0.020	0.016
<i>STAT4</i>	2	rs11685878	0.303	0.458	0.014	0.873	0.924	0.057	0.064	0.105	0.121
<i>STAT4</i>	2	rs7572482	0.961	0.958	0.063	0.735	0.859	0.079	0.730	0.808	0.761
<i>STAT4</i>	2	rs897200	0.219	0.830	0.808	0.313	0.717	0.316	0.484	0.413	0.459
<i>SUMO1</i>	2	rs6717044	0.481	0.319	0.334	0.692	0.694	0.855	0.141	0.152	0.144
<i>SUMO1</i>	2	rs4675272	0.099	0.820	0.947	0.387	0.950	0.994	0.157	0.701	0.770
<i>SUMO1</i>	2	rs6755690	0.260	0.357	0.081	0.402	0.354	0.143	0.483	0.816	0.241
<i>SUMO1</i>	2	rs6709162	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>SUMO1</i>	2	rs3754931	0.735	0.569	0.266	0.252	0.462	0.210	0.427	0.862	0.963
<i>CREB1</i>	2	rs2253206	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>CREB1</i>	2	rs2551640	0.013	0.307	0.176	0.031	0.682	0.314	0.128	0.150	0.367
<i>CREB1</i>	2	rs10932201	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>CREB1</i>	2	rs2254137	0.540	0.068	0.174	0.790	0.083	0.091	0.592	0.547	0.258
<i>SPSB4</i>	3	rs1108693	0.861	0.806	0.416	0.155	0.747	0.721	0.132	0.842	0.193
<i>RNF7</i>	3	rs1980191	0.589	0.380	0.330	0.906	0.729	0.204	0.398	0.273	0.555
<i>RNF7</i>	3	rs6769676	0.672	0.836	0.884	0.439	0.496	0.759	0.862	0.304	0.399
<i>RNF7</i>	3	rs6776205	0.290	0.796	0.630	0.899	0.756	0.524	0.055	1.000	1.000
<i>TFDP2</i>	3	rs2163294	0.611	0.389	0.684	0.666	0.596	0.840	0.198	0.398	0.749
<i>TFDP2</i>	3	rs7642874	0.896	0.868	0.767	0.215	0.843	0.561	0.173	0.537	0.809
<i>TFDP2</i>	3	rs9877536	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>TFDP2</i>	3	rs13065446	0.757	0.939	0.663	0.991	0.722	0.530	0.632	0.466	0.781
<i>ATR</i>	3	rs9816736	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>ATR</i>	3	rs3922730	0.135	0.429	0.702	0.376	0.451	0.519	0.178	0.786	0.657
<i>ATR</i>	3	rs4273389	0.330	0.391	0.489	0.938	0.071	0.063	0.119	0.149	0.023
<i>ATR</i>	3	rs6444085	0.245	0.759	0.303	0.201	0.914	0.306	0.871	0.537	0.781
<i>ATR</i>	3	rs7651071	0.246	0.576	0.855	0.898	0.698	0.627	0.062	0.124	0.052
<i>ATR</i>	3	rs13085998	0.258	0.383	0.669	0.952	0.925	0.483	0.112	0.140	0.175
<i>ATR</i>	3	rs2227928	0.487	0.674	0.352	0.497	0.437	0.304	0.492	0.796	0.895
<i>ATR</i>	3	rs6792259	0.711	0.473	0.811	0.605	0.122	0.380	0.910	0.413	0.700
<i>EMP</i>	4	rs1680073	0.402	0.665	0.191	0.153	0.452	0.261	0.708	0.668	0.247
<i>EMP</i>	4	rs11727167	0.486	0.417	0.629	0.474	0.916	0.390	0.107	0.170	0.249
<i>EMP</i>	4	rs7673398	0.240	0.365	0.305	0.449	0.777	0.377	0.021	0.059	0.175
<i>EMP</i>	4	rs12641735	0.794	0.519	0.619	0.885	0.579	0.682	0.836	0.724	0.895
<i>EMP</i>	4	rs12642410	0.123	0.377	0.603	0.979	0.837	0.484	0.018	0.040	0.133
<i>EMP</i>	4	rs1316393	0.074	0.190	0.135	0.949	0.683	0.081	0.010	0.063	0.114
<i>EMP</i>	4	rs7664474	0.920	0.682	0.811	0.135	0.772	0.863	0.134	0.217	0.490
<i>EMP</i>	4	rs12647145	0.756	0.755	0.818	0.527	0.649	0.911	0.802	0.879	0.479
<i>TNIP2</i>	4	rs9683949	0.298	0.243	0.520	0.235	0.066	0.206	0.900	0.466	0.450
<i>TNIP2</i>	4	rs4690055	0.015	0.191	0.448	0.662	0.584	0.803	0.004	0.001	0.007
<i>TNIP2</i>	4	rs4690060	0.424	1.000	0.746	0.538	1.000	0.356	0.742	1.000	0.543
<i>HTT</i>	4	rs762855	0.286	0.171	0.389	0.373	0.249	0.409	0.489	0.460	0.659
<i>HTT</i>	4	rs2285086	0.816	0.131	0.193	0.146	0.031	0.022	0.132	0.297	0.508
<i>HTT</i>	4	rs10015979	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>HTT</i>	4	rs6446723	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>HTT</i>	4	rs6855981	0.340	0.334	0.275	0.213	0.334	0.287	0.891	0.796	0.872

Gene	Chr	SNP	NHW			NHW FHx			NHW Trios		
			APL	PDT	GenPDT	APL	PDT	GenPDT	APL	PDT	GenPDT
<i>HTT</i>	4	rs4690074	0.688	0.382	0.321	0.684	0.344	0.364	0.925	0.891	0.811
<i>HTT</i>	4	rs363096	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>HTT</i>	4	rs363092	0.728	0.433	0.455	0.355	0.203	0.069	0.552	0.599	0.214
<i>HTT</i>	4	rs362336	0.680	0.944	0.078	0.805	0.937	0.060	0.630	0.768	0.866
<i>HTT</i>	4	rs362331	0.401	1.000	0.875	0.105	0.850	0.936	0.617	0.763	0.828
<i>HTT</i>	4	rs2269478	0.986	0.514	0.831	0.880	0.569	0.537	0.944	0.746	0.433
<i>TLR10</i>	4	rs10776482	0.627	0.317	0.153	0.696	0.933	0.247	0.146	0.080	0.154
<i>TLR10</i>	4	rs11096955	0.850	0.701	0.186	0.850	0.521	0.612	0.614	0.777	0.115
<i>TLR10</i>	4	rs11096957	0.004	0.041	0.148	0.191	0.180	0.462	0.003	0.090	0.203
<i>TLR10</i>	4	rs7658893	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>TLR1</i>	4	rs4833095	0.664	0.662	0.856	0.371	1.000	0.742	0.713	0.355	0.682
<i>TLR1</i>	4	rs5743565	0.690	0.513	0.366	0.677	1.000	0.488	0.811	0.180	0.357
<i>TLR6</i>	4	rs5743818	0.043	0.185	0.504	0.161	0.377	0.703	0.128	0.258	0.135
<i>TLR6</i>	4	rs3821985	0.356	0.246	0.445	0.979	0.936	0.266	0.102	0.014	0.017
<i>TLR6</i>	4	rs5743810	0.194	0.253	0.363	0.313	0.246	0.314	0.433	0.786	0.967
<i>WDR19</i>	4	rs1451821	0.017	0.237	0.412	0.367	0.918	0.983	0.012	0.053	0.135
<i>WDR19</i>	4	rs6815686	0.540	0.867	0.289	0.611	0.463	0.505	0.687	0.484	0.303
<i>WDR19</i>	4	rs9997015	0.152	0.262	0.494	0.104	0.544	0.703	0.864	0.238	0.533
<i>WDR19</i>	4	rs9998591	0.492	0.667	0.499	0.961	0.742	0.686	0.336	0.140	0.364
<i>WDR19</i>	4	rs11096987	0.666	0.368	0.579	0.912	0.480	0.711	0.350	0.572	0.809
<i>WDR19</i>	4	rs3733280	0.017	0.293	0.117	0.307	0.833	0.376	0.015	0.059	0.112
<i>WDR19</i>	4	rs12648082	0.044	0.686	0.631	0.155	0.924	0.759	0.127	0.355	0.646
<i>WDR19</i>	4	rs1057807	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>UBE2K</i>	4	rs3912392	0.929	0.655	0.262	0.830	0.592	0.229	0.912	0.105	0.269
<i>UBE2K</i>	4	rs13122400	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>UBE2K</i>	4	rs12644528	0.575	0.750	0.908	0.278	0.514	0.777	0.916	0.647	0.898
<i>UBE2K</i>	4	rs302947	0.290	0.171	0.389	0.500	0.262	0.319	0.387	0.411	0.113
<i>UBE2K</i>	4	rs305827	0.966	0.898	0.309	0.339	0.440	0.142	0.274	0.042	0.160
<i>UBE2K</i>	4	rs10440307	0.466	0.761	0.434	0.359	0.513	0.697	0.977	0.405	0.277
<i>UBE2K</i>	4	rs4263408	0.556	0.657	0.892	0.032	0.211	0.508	0.089	0.237	0.515
<i>CARMA1</i>	7	rs11982651	0.607	0.570	0.829	0.346	0.123	0.222	0.753	0.140	0.114
<i>CARMA1</i>	7	rs1713911	0.518	1.000	0.973	0.921	0.689	0.897	0.386	0.612	0.889
<i>CARMA1</i>	7	rs1476636	0.521	0.109	0.311	0.196	0.022	0.090	0.583	0.411	0.625
<i>CARMA1</i>	7	rs4722276	0.985	0.690	0.091	0.633	0.698	0.237	0.731	0.889	0.344
<i>CARMA1</i>	7	rs12538346	0.598	0.357	0.650	0.584	0.190	0.290	0.881	0.789	0.484
<i>CARMA1</i>	7	rs10236776	0.026	0.049	0.121	0.024	0.050	0.116	0.356	0.612	0.692
<i>CARMA1</i>	7	rs4722356	0.911	0.945	0.812	0.364	0.622	0.438	0.217	0.529	0.565
<i>RBAK</i>	7	rs7805748	0.381	0.758	0.820	0.278	0.454	0.719	0.847	0.586	0.241
<i>RBAK</i>	7	rs10238244	0.175	0.080	0.169	0.136	0.048	0.093	0.875	1.000	0.333
<i>RBAK</i>	7	rs7778444	0.767	0.241	0.444	0.988	0.284	0.345	0.587	0.631	0.805
<i>TRIAD3</i>	7	rs852374	0.608	0.196	0.426	0.624	0.325	0.558	0.836	0.387	0.716
<i>TRIAD3</i>	7	rs13246406	0.275	0.544	0.765	0.092	0.371	0.620	0.962	0.724	0.921
<i>TRIAD3</i>	7	rs852522	0.916	0.689	0.356	0.905	0.385	0.550	0.781	0.612	0.404
<i>TRIAD3</i>	7	rs13247447	0.130	0.645	0.690	0.214	0.365	0.594	0.327	0.564	0.717
<i>TRIAD3</i>	7	rs852417	0.093	0.317	0.513	0.174	1.000	1.000	0.291	0.317	0.317
<i>TRIAD3</i>	7	rs3823681	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>TRIAD3</i>	7	rs3779092	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>TRIAD3</i>	7	rs852394	0.362	0.159	0.265	0.460	0.330	0.306	0.564	0.258	0.540

Gene	Chr	SNP	NHW			NHW FHx			NHW Trios		
			APL	PDT	GenPDT	APL	PDT	GenPDT	APL	PDT	GenPDT
<i>TRIAD3</i>	7	rs1468996	0.411	0.900	0.925	0.573	0.944	0.743	0.601	0.680	0.071
<i>TRIAD3</i>	7	rs2302907	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>TRIAD3</i>	7	rs13239194	0.571	0.404	0.452	0.429	1.000	0.488	0.131	0.170	0.390
<i>TRIAD3</i>	7	rs13237614	0.755	0.772	0.830	0.192	0.732	0.636	0.077	0.273	0.518
<i>TRIAD3</i>	7	rs2112006	0.801	0.137	0.446	0.528	0.052	0.289	0.872	1.000	0.920
<i>TRIAD3</i>	7	rs11771172	0.012	0.127	0.121	0.006	0.336	0.361	0.659	0.189	0.233
<i>TRIAD3</i>	7	rs6971918	0.730	0.408	0.773	0.513	0.320	0.685	0.802	0.886	0.974
<i>TRIAD3</i>	7	rs10257204	0.680	0.290	0.352	0.404	0.768	0.614	0.254	0.149	0.268
<i>TRIAD3</i>	7	rs6967635	0.421	0.502	0.813	0.482	0.368	0.712	0.648	0.763	0.910
<i>TRIAD3</i>	7	rs2017620	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>TNFSF13B</i>	13	rs9514828	0.619	0.536	0.760	0.969	0.216	0.345	0.523	0.453	0.137
<i>TNFSF13B</i>	13	rs8181791	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>TNFSF13B</i>	13	rs10508198	0.329	0.644	0.777	0.628	0.912	0.753	0.421	0.317	0.214
<i>TNFSF13B</i>	13	rs9520835	0.004	0.009	0.023	0.005	0.113	0.166	0.218	0.022	0.083
<i>TNFSF13B</i>	13	rs1224163	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>ING1</i>	13	rs4773240	0.388	0.645	0.538	0.249	0.433	0.697	0.837	0.493	0.309
<i>ING1</i>	13	rs1441043	0.038	0.788	0.118	0.551	0.739	0.407	0.020	0.366	0.184
<i>ING1</i>	13	rs6492308	0.166	0.667	0.877	0.174	0.916	0.910	0.653	0.555	0.866
<i>TFDP1</i>	13	rs7325214	0.428	0.902	0.367	0.846	0.826	0.786	0.283	0.569	0.141
<i>TFDP1</i>	13	rs4150703	0.289	0.581	0.140	0.348	0.383	0.171	0.523	0.732	0.638
<i>TFDP1</i>	13	rs9577595	0.805	0.504	0.098	0.480	0.269	0.162	0.789	0.706	0.475
<i>TFDP1</i>	13	rs12428926	0.247	1.000	0.812	0.605	0.331	0.232	0.022	0.114	0.126
<i>TFDP1</i>	13	rs4150832	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>TNFRSF11A</i>	18	rs2981007	0.362	0.854	0.209	0.086	0.947	0.297	0.575	0.758	0.419
<i>TNFRSF11A</i>	18	rs4941125	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>TNFRSF11A</i>	18	rs7239261	0.302	0.419	0.335	0.211	0.461	0.319	0.784	0.732	0.932
<i>TNFRSF11A</i>	18	rs4263037	0.484	0.887	0.972	0.520	0.933	0.682	0.737	0.895	0.207
<i>TNFRSF11A</i>	18	rs7236060	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>TNFRSF11A</i>	18	rs8094884	0.292	1.000	0.786	0.607	0.865	0.275	0.362	0.768	0.452
<i>TNFRSF11A</i>	18	rs8089829	0.041	0.660	0.178	0.048	0.558	0.623	0.422	0.879	0.100
<i>TNFRSF11A</i>	18	rs12959396	0.565	0.687	0.335	0.204	0.871	0.708	0.736	0.500	0.229
<i>TNFRSF11A</i>	18	rs9646629	0.240	0.059	0.070	0.090	0.103	0.135	0.754	0.317	0.317
<i>TNFRSF11A</i>	18	rs2957125	0.238	0.088	0.197	0.542	0.116	0.510	0.307	0.423	0.081
<i>SOCS6</i>	18	rs7230661	0.135	0.777	0.338	0.308	0.398	0.161	0.265	0.439	0.749
<i>SOCS6</i>	18	rs713130	0.379	0.293	0.176	0.379	0.225	0.456	0.658	1.000	0.021
<i>SOCS6</i>	18	rs2053420	0.037	0.041	0.062	0.016	0.030	0.080	0.806	0.691	0.695
<i>NFAT2</i>	18	rs9962479	0.942	0.555	0.391	0.430	0.347	0.261	0.454	0.662	0.890
<i>NFAT2</i>	18	rs8090692	0.892	0.172	0.308	0.649	0.365	0.648	0.714	0.267	0.290
<i>NFAT2</i>	18	rs2036892	0.585	0.163	0.401	0.862	0.086	0.136	0.379	0.668	0.429
<i>NFAT2</i>	18	rs4799055	0.957	0.475	0.484	0.523	0.686	0.280	0.445	0.332	0.484
<i>NFAT2</i>	18	rs8097537	0.852	0.364	0.173	0.965	0.160	0.071	0.731	0.500	0.807
<i>NFAT2</i>	18	rs12608349	0.848	0.710	0.761	0.913	0.480	0.758	0.688	0.606	0.171
<i>NFAT2</i>	18	rs2290154	0.397	0.490	0.482	0.688	0.847	0.542	0.073	0.241	0.369
<i>NFAT2</i>	18	rs7227107	0.412	0.493	0.040	0.683	1.000	0.189	0.126	0.206	0.169
<i>NFAT2</i>	18	rs370989	0.458	0.020	0.062	0.495	0.013	0.036	0.700	0.896	0.979
<i>NFAT2</i>	18	rs1667673	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>NFAT2</i>	18	rs1660139	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>NFAT2</i>	18	rs177820	0.684	0.620	0.833	0.561	0.871	0.976	0.230	0.385	0.445

Gene	Chr	SNP	NHW			NHW FHx			NHW Trios		
			APL	PDT	GenPDT	APL	PDT	GenPDT	APL	PDT	GenPDT
<i>NFAT2</i>	18	rs3894049	0.788	0.041	0.166	0.560	0.042	0.163	0.827	0.593	0.527
<i>NFAT2</i>	18	rs183374	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Table B3. Association of apoptotic genes with clubfoot in Hispanics.

Gene	Chr	SNP	Hispanic			Hispanic FHx			Hispanic Trios		
			APL	PDT	GenPDT	APL	PDT	GenPDT	APL	PDT	GenPDT
ZAK	2	rs989531	0.552	0.316	0.448	0.359	0.262	0.329	0.938	0.866	0.511
ZAK	2	rs6433395	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
ZAK	2	rs6759787	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
ZAK	2	rs17302977	0.273	0.328	0.502	0.288	0.562	0.393	0.588	0.178	0.226
ZAK	2	rs3769192	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
ZAK	2	rs4344898	0.156	0.079	0.152	0.385	0.340	0.564	0.310	0.103	0.215
ZAK	2	rs13032010	0.390	0.063	0.231	0.933	0.237	0.143	0.351	0.152	0.183
ZAK	2	rs1837470	0.981	0.371	0.537	0.386	0.380	0.596	0.518	0.786	0.897
ZAK	2	rs4972533	0.328	0.702	0.672	0.486	0.277	0.493	0.085	0.317	0.461
ZAK	2	rs2028382	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
ZAK	2	rs11685001	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
ZAK	2	rs11686011	0.797	0.406	0.720	0.278	0.144	0.250	0.657	0.876	0.723
ZAK	2	rs12618933	0.988	0.496	0.690	0.391	0.463	0.579	0.624	1.000	1.000
ATF2/CREB2	2	rs212352	0.046	0.069	0.063	0.500	0.254	0.236	0.070	0.083	0.175
ATF2/CREB2	2	rs2698545	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
ATF2/CREB2	2	rs10930693	0.525	0.423	0.768	0.815	0.632	0.915	0.290	0.466	0.774
CERKL	2	rs1047307	0.825	0.424	0.549	0.809	0.340	0.445	0.763	0.858	0.441
CERKL	2	rs11680383	0.325	0.448	0.702	0.281	0.456	0.668	0.664	0.873	0.796
CERKL	2	rs895901	0.327	0.190	0.333	0.899	0.831	0.289	0.196	0.019	0.110
CERKL	2	rs10445770	0.342	0.861	0.871	0.038	0.225	0.373	0.740	0.310	0.100
CERKL	2	rs1992394	0.963	1.000	0.963	0.494	0.594	0.742	0.535	0.160	0.412
CERKL	2	rs935087	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
CERKL	2	rs1866888	0.751	0.562	0.830	0.937	0.547	0.808	0.749	0.893	0.958
GULP1	2	rs10931346	0.345	0.353	0.262	0.788	0.406	0.382	0.285	0.655	0.627
GULP1	2	rs7593546	0.726	0.603	0.850	0.367	0.480	0.709	0.827	0.891	0.799
GULP1	2	rs4396679	0.698	1.000	0.302	0.535	0.697	0.235	0.932	0.446	0.738
GULP1	2	rs7586390	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
GULP1	2	rs12624002	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
GULP1	2	rs12474692	0.987	0.424	0.099	0.921	0.935	0.128	0.964	0.114	0.285
GULP1	2	rs10931359	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
GULP1	2	rs6724428	0.622	0.592	0.777	0.946	0.413	0.680	0.601	0.777	0.413
GULP1	2	rs11685321	0.786	0.301	0.573	0.110	0.460	0.656	0.534	0.013	0.030
GULP1	2	rs13034731	0.418	0.281	0.589	0.332	0.876	0.943	0.761	0.180	0.364
GULP1	2	rs1354905	0.204	0.043	0.243	0.161	0.025	0.246	0.692	0.492	0.431
STAT1	2	rs12468579	0.872	0.286	0.452	0.286	0.077	0.212	0.379	0.873	0.975
STAT1	2	rs13395505	0.155	0.007	0.040	0.835	0.251	0.646	0.089	0.013	0.050
STAT1	2	rs2280233	0.770	0.604	0.616	0.742	0.748	0.454	0.973	0.662	0.913
STAT1	2	rs7562024	0.144	0.491	0.010	0.986	0.758	0.182	0.105	0.170	0.025
STAT1	2	rs6751855	0.959	0.292	0.381	0.369	0.519	0.763	0.461	0.317	0.155
STAT4	2	rs925847	0.429	0.092	0.072	0.016	0.096	0.136	0.537	0.578	0.419
STAT4	2	rs16833215	0.765	0.922	0.222	0.064	0.206	0.368	0.386	0.385	0.072
STAT4	2	rs1517352	0.617	0.302	0.665	0.813	0.493	0.474	0.685	0.439	0.469
STAT4	2	rs16833249	0.516	0.102	0.039	0.685	0.221	0.305	0.650	0.262	0.026
STAT4	2	rs11693480	0.568	1.000	0.298	0.781	0.144	0.216	0.380	0.228	0.088
STAT4	2	rs12463658	0.209	0.906	0.116	0.544	0.847	0.269	0.314	0.763	0.418
STAT4	2	rs4853543	0.400	0.134	0.330	0.109	0.159	0.462	0.981	0.492	0.598
STAT4	2	rs4341967	0.808	1.000	0.169	0.759	0.851	0.192	0.919	0.758	0.611

Gene	Chr	SNP	Hisp.			Hisp. FHx			Hisp. Trios		
			APL	PDT	GenPDT	APL	PDT	GenPDT	APL	PDT	GenPDT
<i>STAT4</i>	2	rs6738544	0.300	0.093	0.330	0.106	0.044	0.212	0.916	0.746	0.260
<i>STAT4</i>	2	rs7574909	0.168	0.095	0.283	0.383	0.083	0.153	0.280	0.547	0.595
<i>STAT4</i>	2	rs2356350	0.760	0.914	0.670	0.806	0.901	0.472	0.873	1.000	1.000
<i>STAT4</i>	2	rs11685878	0.751	0.117	0.087	0.724	0.128	0.149	0.444	0.537	0.038
<i>STAT4</i>	2	rs7572482	0.141	0.325	0.053	0.381	0.900	0.478	0.202	0.128	0.049
<i>STAT4</i>	2	rs897200	0.443	0.393	0.434	0.361	0.345	0.474	0.772	0.789	0.698
<i>SUMO1</i>	2	rs6717044	0.714	0.927	0.614	0.539	0.816	0.170	0.973	0.882	0.835
<i>SUMO1</i>	2	rs4675272	0.693	0.933	0.484	0.152	0.732	0.927	0.642	0.806	0.214
<i>SUMO1</i>	2	rs6755690	0.179	0.755	0.292	0.921	0.051	0.040	0.113	0.071	0.051
<i>SUMO1</i>	2	rs6709162	0.898	0.612	0.244	0.585	0.178	0.122	0.577	0.307	0.651
<i>SUMO1</i>	2	rs3754931	0.237	0.510	0.735	0.032	0.106	0.205	0.920	0.257	0.492
<i>CREB1</i>	2	rs2253206	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>CREB1</i>	2	rs2551640	0.758	0.845	0.922	0.607	0.722	0.716	0.334	0.384	0.315
<i>CREB1</i>	2	rs10932201	0.193	0.922	0.891	0.658	0.086	0.283	0.061	0.286	0.356
<i>CREB1</i>	2	rs2254137	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>SPSB4</i>	3	rs1108693	0.159	0.022	0.098	0.256	0.050	0.090	0.349	0.237	0.121
<i>RNF7</i>	3	rs1980191	0.383	0.052	0.187	0.351	0.128	0.421	0.681	0.228	0.156
<i>RNF7</i>	3	rs6769676	0.225	0.484	0.526	0.796	0.670	0.477	0.168	0.578	0.829
<i>RNF7</i>	3	rs6776205	0.962	0.925	0.986	0.746	0.696	0.776	0.763	0.586	0.809
<i>TFDP2</i>	3	rs2163294	0.603	0.603	0.840	0.258	0.325	0.561	1.000	0.593	0.092
<i>TFDP2</i>	3	rs7642874	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>TFDP2</i>	3	rs9877536	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>TFDP2</i>	3	rs13065446	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>ATR</i>	3	rs9816736	0.196	0.178	0.112	0.883	0.469	0.146	0.179	0.139	0.072
<i>ATR</i>	3	rs3922730	0.425	0.773	0.235	0.681	0.414	0.254	0.498	0.221	0.354
<i>ATR</i>	3	rs4273389	0.133	0.399	0.135	0.022	0.433	0.728	0.653	0.647	0.098
<i>ATR</i>	3	rs6440085	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>ATR</i>	3	rs7651071	0.906	0.192	0.141	0.077	0.280	0.281	0.230	0.456	0.468
<i>ATR</i>	3	rs13085998	0.617	0.325	0.459	0.240	0.387	0.588	0.730	0.602	0.308
<i>ATR</i>	3	rs2227928	0.733	0.356	0.531	0.883	0.174	0.289	0.615	0.131	0.231
<i>ATR</i>	3	rs6792259	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>EMP</i>	4	rs1680073	0.633	0.651	0.874	0.282	0.612	0.843	0.816	0.879	0.773
<i>EMP</i>	4	rs11727167	0.809	0.235	0.584	0.503	0.132	0.426	0.824	0.793	0.624
<i>EMP</i>	4	rs7673398	0.862	0.916	0.448	0.611	0.586	0.418	0.847	0.612	0.801
<i>EMP</i>	4	rs12641735	0.077	0.048	0.051	0.726	0.201	0.399	0.045	0.131	0.080
<i>EMP</i>	4	rs12642410	0.328	0.035	0.218	0.125	0.040	0.325	0.868	0.264	0.309
<i>EMP</i>	4	rs1316393	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>EMP</i>	4	rs7664474	0.577	0.166	0.478	0.091	0.027	0.302	0.741	1.000	0.486
<i>EMP</i>	4	rs12647145	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>TNIP2</i>	4	rs9683949	0.603	0.652	0.278	0.645	0.827	0.360	0.756	0.631	0.629
<i>TNIP2</i>	4	rs4690055	0.783	0.216	0.420	0.942	1.000	0.683	0.726	0.103	0.252
<i>TNIP2</i>	4	rs4690060	0.329	1.000	0.681	0.707	0.505	0.678	0.198	0.606	0.749
<i>HTT</i>	4	rs762855	0.010	0.211	0.469	0.053	0.078	0.211	0.105	0.842	0.921
<i>HTT</i>	4	rs2285086	0.682	0.547	0.602	0.573	0.397	0.642	0.961	0.670	0.418
<i>HTT</i>	4	rs10015979	0.484	0.838	0.980	0.956	0.889	0.588	0.503	0.655	0.548
<i>HTT</i>	4	rs6446723	0.042	0.365	0.172	<0.001	0.330	0.587	0.654	0.752	0.107
<i>HTT</i>	4	rs6855981	0.265	0.710	0.758	0.240	0.633	0.217	0.656	1.000	0.657
<i>HTT</i>	4	rs4690074	0.799	0.169	0.246	0.590	0.513	0.528	0.518	0.128	0.018

Gene	Chr	SNP	Hispanic			Hispanic FHx			Hispanic Trios		
			APL	PDT	GenPDT	APL	PDT	GenPDT	APL	PDT	GenPDT
<i>HTT</i>	4	rs363096	0.567	0.806	0.223	0.913	0.862	0.754	0.561	0.602	0.247
<i>HTT</i>	4	rs363092	0.370	0.228	0.165	0.819	0.268	0.322	0.340	0.622	0.408
<i>HTT</i>	4	rs362336	0.994	0.199	0.355	0.406	0.096	0.147	0.515	0.622	0.703
<i>HTT</i>	4	rs362331	0.936	0.686	0.356	0.564	0.907	0.432	0.583	0.549	0.757
<i>HTT</i>	4	rs2269478	0.144	0.384	0.462	0.363	0.637	0.665	0.239	0.398	0.512
<i>TLR10</i>	4	rs10776482	0.153	0.480	0.552	0.133	0.399	0.464	0.535	1.000	0.878
<i>TLR10</i>	4	rs11096955	0.118	0.547	0.696	0.022	0.683	0.879	0.648	0.297	0.445
<i>TLR10</i>	4	rs11096957	0.932	0.942	0.537	0.763	0.859	0.690	0.867	0.710	0.761
<i>TLR10</i>	4	rs7658893	0.481	0.251	0.183	0.750	0.245	0.327	0.529	0.900	0.376
<i>TLR1</i>	4	rs4833095	0.670	0.180	0.129	0.296	0.458	0.735	0.268	0.182	0.013
<i>TLR1</i>	4	rs5743565	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>TLR6</i>	4	rs5743818	0.444	0.289	0.543	0.610	0.772	0.902	0.548	0.038	0.095
<i>TLR6</i>	4	rs3821985	0.665	0.458	0.722	0.490	0.742	0.318	0.301	0.384	0.448
<i>TLR6</i>	4	rs5743810	0.379	0.015	0.088	0.192	0.179	0.243	0.053	0.033	0.112
<i>WDR19</i>	4	rs1451821	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>WDR19</i>	4	rs6815686	0.186	0.140	0.248	0.054	0.586	0.818	0.707	0.096	0.131
<i>WDR19</i>	4	rs9997015	0.248	0.029	0.045	0.271	0.067	0.142	0.414	0.216	0.230
<i>WDR19</i>	4	rs9998591	0.622	0.701	0.769	0.139	0.933	0.664	0.629	0.433	0.796
<i>WDR19</i>	4	rs11096987	0.664	0.207	0.379	0.701	0.302	0.576	0.455	0.446	0.236
<i>WDR19</i>	4	rs3733280	0.437	0.820	0.850	0.502	0.281	0.463	0.150	0.199	0.385
<i>WDR19</i>	4	rs12648082	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>WDR19</i>	4	rs1057807	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>UBE2K</i>	4	rs3912392	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>UBE2K</i>	4	rs13122400	0.131	0.187	0.134	0.651	0.535	0.512	0.113	0.157	0.169
<i>UBE2K</i>	4	rs12644528	0.203	0.383	0.288	0.771	0.706	0.744	0.205	0.307	0.235
<i>UBE2K</i>	4	rs302947	0.160	0.150	0.332	0.200	0.375	0.530	0.424	0.216	0.453
<i>UBE2K</i>	4	rs305827	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>UBE2K</i>	4	rs10440307	0.925	0.330	0.112	0.175	0.317	0.178	0.338	0.670	0.507
<i>UBE2K</i>	4	rs4263408	0.657	0.678	0.888	0.244	0.756	0.882	0.209	0.267	0.351
<i>CARMA1</i>	7	rs11982651	0.158	0.302	0.573	0.923	0.746	0.893	0.091	0.285	0.582
<i>CARMA1</i>	7	rs1713911	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>CARMA1</i>	7	rs1476636	0.815	0.052	0.177	0.232	0.052	0.193	0.626	0.516	0.601
<i>CARMA1</i>	7	rs4722276	0.814	0.537	0.521	0.260	0.371	0.570	0.563	1.000	0.654
<i>CARMA1</i>	7	rs12538346	0.603	0.082	0.322	0.275	0.100	0.323	0.873	0.522	0.484
<i>CARMA1</i>	7	rs10236776	0.265	0.211	0.444	0.407	0.216	0.336	0.506	0.758	0.258
<i>CARMA1</i>	7	rs4722356	0.109	0.243	0.031	0.090	0.924	0.391	0.483	0.028	0.048
<i>RBAK</i>	7	rs7805748	0.189	0.884	0.724	0.602	0.601	0.816	0.242	0.354	0.134
<i>RBAK</i>	7	rs10238244	0.640	0.258	0.494	0.447	0.144	0.284	0.941	0.686	0.905
<i>RBAK</i>	7	rs7778444	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>TRIAD3</i>	7	rs852374	0.005	0.003	0.020	0.013	0.020	0.087	0.058	0.063	0.223
<i>TRIAD3</i>	7	rs13246406	0.747	1.000	1.000	0.240	0.619	0.691	0.266	0.446	0.755
<i>TRIAD3</i>	7	rs852522	0.066	0.012	0.042	0.006	0.017	0.116	0.759	0.297	0.195
<i>TRIAD3</i>	7	rs13247447	0.359	0.384	0.383	0.275	0.446	0.800	0.655	0.655	0.181
<i>TRIAD3</i>	7	rs852417	0.612	0.317	0.513	0.440	1.000	1.000	0.272	0.317	0.317
<i>TRIAD3</i>	7	rs3823681	0.429	0.928	0.991	0.848	0.895	0.853	0.317	1.000	0.739
<i>TRIAD3</i>	7	rs3779092	0.594	0.912	0.572	0.996	0.516	0.453	0.543	0.446	0.681
<i>TRIAD3</i>	7	rs852394	0.515	0.878	0.646	0.136	0.626	0.378	0.775	0.710	0.850
<i>TRIAD3</i>	7	rs1468996	0.599	1.000	0.831	0.211	1.000	0.616	0.913	1.000	1.000

Gene	Chr	SNP	Hisp.			Hisp. FHx			Hisp. Trios		
			APL	PDT	GenPDT	APL	PDT	GenPDT	APL	PDT	GenPDT
<i>TRIAD3</i>	7	rs2302907	0.158	0.384	0.713	0.072	0.505	0.820	0.487	0.553	0.805
<i>TRIAD3</i>	7	rs13239194	0.578	0.535	0.670	0.891	0.425	0.487	0.445	1.000	0.203
<i>TRIAD3</i>	7	rs13237614	0.391	0.155	0.248	0.682	0.361	0.550	0.180	0.257	0.402
<i>TRIAD3</i>	7	rs2112006	0.933	0.508	0.185	0.910	0.796	0.379	0.889	0.392	0.279
<i>TRIAD3</i>	7	rs11771172	0.558	0.722	0.090	0.209	0.310	0.584	0.111	0.134	0.050
<i>TRIAD3</i>	7	rs6971918	0.732	0.652	0.445	0.817	0.612	0.283	0.785	0.898	0.980
<i>TRIAD3</i>	7	rs10257204	0.773	0.909	0.544	0.804	0.862	0.754	0.789	0.763	0.683
<i>TRIAD3</i>	7	rs6967635	0.882	0.459	0.507	0.455	0.183	0.279	0.488	0.500	0.725
<i>TRIAD3</i>	7	rs2017620	0.612	0.157	0.363	0.311	0.170	0.385	0.859	0.578	0.860
<i>TNFSF13B</i>	13	rs9514828	0.218	0.684	0.593	0.159	0.763	0.866	0.646	0.782	0.622
<i>TNFSF13B</i>	13	rs8181791	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>TNFSF13B</i>	13	rs10508198	0.353	0.579	0.690	0.904	0.267	0.252	0.246	0.578	0.177
<i>TNFSF13B</i>	13	rs9520835	0.849	0.395	0.075	0.423	0.066	0.120	0.808	0.655	0.425
<i>TNFSF13B</i>	13	rs1224163	0.911	0.243	0.462	0.371	0.269	0.416	0.403	0.623	0.879
<i>ING1</i>	13	rs4773240	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>ING1</i>	13	rs1441043	0.117	0.056	0.157	0.722	0.157	0.337	0.056	0.189	0.380
<i>ING1</i>	13	rs6492308	0.236	0.164	0.227	0.337	0.311	0.416	0.448	0.217	0.357
<i>TFDP1</i>	13	rs7325214	0.688	0.579	0.414	0.252	0.274	0.175	0.178	0.752	0.899
<i>TFDP1</i>	13	rs4150703	0.139	0.070	0.016	0.929	0.819	0.269	0.053	0.028	0.041
<i>TFDP1</i>	13	rs9577595	0.610	0.630	0.513	0.315	0.352	0.228	0.870	0.456	0.695
<i>TFDP1</i>	13	rs12428926	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>TFDP1</i>	13	rs4150832	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>TNFRSF11A</i>	18	rs2981007	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>TNFRSF11A</i>	18	rs4941125	0.025	0.475	0.635	<0.001	0.608	0.810	0.598	0.617	0.777
<i>TNFRSF11A</i>	18	rs7239261	0.007	0.075	0.179	0.103	0.353	0.636	0.033	0.128	0.276
<i>TNFRSF11A</i>	18	rs4263037	0.337	0.454	0.765	0.307	0.354	0.612	0.597	0.895	0.979
<i>TNFRSF11A</i>	18	rs7236060	0.654	0.296	0.513	0.752	0.592	0.391	0.816	0.332	0.096
<i>TNFRSF11A</i>	18	rs8094884	0.071	0.010	0.111	0.042	0.059	0.435	0.448	0.066	0.185
<i>TNFRSF11A</i>	18	rs8089829	0.789	0.840	0.922	0.875	0.686	0.859	0.836	0.879	0.976
<i>TNFRSF11A</i>	18	rs12959396	0.765	0.796	0.584	0.111	0.793	0.309	0.557	0.569	0.774
<i>TNFRSF11A</i>	18	rs9646629	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>TNFRSF11A</i>	18	rs2957125	0.463	0.842	0.533	0.635	0.486	0.320	0.284	0.117	0.286
<i>SOCS6</i>	18	rs7230661	0.600	0.629	0.496	0.843	1.000	0.226	0.412	0.453	0.701
<i>SOCS6</i>	18	rs713130	0.727	0.689	0.625	0.730	0.276	0.202	0.821	0.555	0.866
<i>SOCS6</i>	18	rs2053420	0.125	0.191	0.387	0.152	0.739	0.782	0.424	0.118	0.153
<i>NFATC1/NFAT2</i>	18	rs9962479	0.924	0.297	0.204	0.275	0.083	0.083	0.242	0.763	0.695
<i>NFATC1/NFAT2</i>	18	rs8090692	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>NFATC1/NFAT2</i>	18	rs2036892	0.996	0.706	0.273	0.856	0.564	0.624	0.944	0.317	0.271
<i>NFATC1/NFAT2</i>	18	rs4799055	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<i>NFATC1/NFAT2</i>	18	rs8097537	0.025	<0.001	0.002	0.183	0.433	0.752	0.053	<0.001	0.001
<i>NFATC1/NFAT2</i>	18	rs12608349	0.102	0.061	0.001	0.552	0.884	0.027	0.021	0.016	0.005
<i>NFATC1/NFAT2</i>	18	rs2290154	0.877	0.928	0.922	0.540	0.366	0.680	0.766	0.574	0.840
<i>NFATC1/NFAT2</i>	18	rs7227107	0.958	0.884	0.423	0.808	0.414	0.574	0.817	0.876	0.635
<i>NFATC1/NFAT2</i>	18	rs370989	0.003	0.003	0.003	0.001	0.274	0.164	0.077	0.003	0.009
<i>NFATC1/NFAT2</i>	18	rs1667673	0.123	0.206	0.347	0.291	0.297	0.366	0.249	0.456	0.761
<i>NFATC1/NFAT2</i>	18	rs1660139	0.690	0.302	0.537	0.657	0.144	0.073	0.499	0.803	0.613
<i>NFATC1/NFAT2</i>	18	rs177820	0.399	0.317	0.550	0.062	0.132	0.186	0.709	0.655	0.761
<i>NFATC1/NFAT2</i>	18	rs3894049	<0.001	0.001	0.004	0.016	0.670	0.935	0.007	0.000	0.000

Gene	Chr	SNP	Hispanic			Hispanic FHx			Hispanic Trios		
			APL	PDT	GenPDT	APL	PDT	GenPDT	APL	PDT	GenPDT
<i>NFATC1/NFAT2</i>	18	rs183374	0.588	0.212	0.430	0.199	0.615	0.868	0.133	0.172	0.422

Table B4. Gene-gene interactions of clubfoot deletion region apoptotic genes p<0.01 in NHW.

Gene 1	SNP 1	Gene 2	SNP 2	p-value
<i>ATF2/CREB2</i>	rs10930693	CERKL	rs11680383	0.0024
<i>ATF2/CREB2</i>	rs10930693	HTT	rs2269478	0.0091
<i>ATF2/CREB2</i>	rs2698545	TNFSF13B	rs8181791	0.0082
<i>ATR</i>	rs13085998	NFATC1/NFAT2	rs4799055	0.0029
<i>ATR</i>	rs13085998	NFATC1/NFAT2	rs8097537	0.0073
<i>ATR</i>	rs13085998	TNIP2	rs4690060	0.0046
<i>ATR</i>	rs13085998	TRIAD3	rs13246406	0.0088
<i>ATR</i>	rs2227928	TRIAD3	rs852394	0.0098
<i>ATR</i>	rs6440085	TRIAD3	rs852417	0.0079
<i>ATR</i>	rs13085998	UBE2K	rs302947	0.0044
<i>ATR</i>	rs6792259	UBE2K	rs302947	0.0094
<i>ATR</i>	rs7651071	UBE2K	rs302947	0.0042
<i>CARD11/CARMA1</i>	rs12538346	ING1	rs4773240	0.0074
<i>CARD11/CARMA1</i>	rs11982651	NFATC1/NFAT2	rs2290154	0.0054
<i>CARD11/CARMA1</i>	rs1713911	TNFSF13B	rs8181791	0.002
<i>CERKL</i>	rs1866888	HTT	rs6855981	0.0035
<i>CERKL</i>	rs1866888	EMP	rs12641735	0.0011
<i>CERKL</i>	rs11680383	NFATC1/NFAT2	rs1660139	0.0091
<i>CERKL</i>	rs1866888	TFDP1	rs4150832	0.0015
<i>CERKL</i>	rs1047307	TLR1	rs5743565	0.009
<i>CERKL</i>	rs895901	TLR6	rs3821985	0.007
<i>CERKL</i>	rs11680383	TNFRSF11A	rs9646629	0.0068
<i>CREB1</i>	rs2253206	ATR	rs6440085	0.0037
<i>CREB1</i>	rs2551640	HTT	rs2285086	0.0002
<i>CREB1</i>	rs10932201	NFATC1/NFAT2	rs2290154	0.0057
<i>CREB1</i>	rs2254137	NFATC1/NFAT2	rs3894049	0.0068
<i>CREB1</i>	rs2551640	NFATC1/NFAT2	rs3894049	0.0044
<i>CREB1</i>	rs2254137	TLR10	rs11096955	0.0058
<i>GULP1</i>	rs10931359	ING1	rs4773240	0.01
<i>GULP1</i>	rs13034731	STAT4	rs1517352	0.0076
<i>GULP1</i>	rs10931346	TFDP2	rs13065446	0.0082
<i>GULP1</i>	rs12624002	TFDP2	rs13065446	0.0092
<i>GULP1</i>	rs13034731	TFDP2	rs13065446	0.0031
<i>GULP1</i>	rs13034731	TFDP2	rs7642874	0.0019
<i>GULP1</i>	rs7586390	TNFRSF11A	rs7239261	0.0078
<i>GULP1</i>	rs1354905	TNFSF13B	rs10508198	0.0004
<i>GULP1</i>	rs13034731	TRIAD3	rs10257204	0.0063
<i>GULP1</i>	rs1354905	UBE2K	rs4263408	0.0067
<i>GULP1</i>	rs13034731	WDR19	rs12648082	0.0048
<i>GULP1</i>	rs12624002	WDR19	rs9998591	0.0015
<i>GULP1</i>	rs7586390	WDR19	rs9998591	0.0063
<i>HTT</i>	rs2285086	TLR1	rs4833095	0.0096
<i>HTT</i>	rs2269478	TRIAD3	rs6967635	0.0044
<i>HTT</i>	rs762855	TRIAD3	rs6967635	0.0043

Gene 1	SNP 1	Gene 2	SNP 2	p-value
<i>HTT</i>	rs2269478	TRIAD3	rs6971918	0.0045
<i>HTT</i>	rs363092	TRIAD3	rs852394	0.01
<i>HTT</i>	rs6855981	TRIAD3	rs852394	0.01
<i>HTT</i>	rs10015979	TRIAD3	rs852417	0.0021
<i>HTT</i>	rs362331	TRIAD3	rs852417	0.003
<i>HTT</i>	rs362336	UBE2K	rs302947	0.001
<i>HTT</i>	rs2285086	WDR19	rs3733280	0.0043
<i>ING1</i>	rs1441043	NFATC1/NFAT2	rs8090692	0.0073
<i>EMP</i>	rs12647145	HTT	rs362336	0.0043
<i>EMP</i>	rs1316393	HTT	rs6855981	0.0081
<i>EMP</i>	rs1680073	EMP	rs11727167	0.0091
<i>EMP</i>	rs1680073	EMP	rs7664474	0.0067
<i>EMP</i>	rs7664474	NFATC1/NFAT2	rs183374	0.0032
<i>EMP</i>	rs1316393	NFATC1/NFAT2	rs2036892	0.0049
<i>EMP</i>	rs12641735	NFATC1/NFAT2	rs3894049	0.0015
<i>EMP</i>	rs7673398	TLR10	rs11096955	0.0032
<i>EMP</i>	rs11727167	TLR10	rs11096957	0.0063
<i>EMP</i>	rs12642410	TLR10	rs11096957	0.0022
<i>EMP</i>	rs1316393	TLR10	rs11096957	0.0026
<i>EMP</i>	rs7664474	TLR10	rs11096957	0.007
<i>EMP</i>	rs7673398	TLR10	rs11096957	0.005
<i>EMP</i>	rs12641735	TNFRSF11A	rs8089829	0.0043
<i>EMP</i>	rs12642410	TRIAD3	rs13246406	0.0042
<i>EMP</i>	rs7664474	TRIAD3	rs852417	0.0054
<i>EMP</i>	rs1680073	UBE2K	rs10440307	0.0013
<i>EMP</i>	rs12641735	WDR19	rs11096987	0.0037
<i>EMP</i>	rs12641735	WDR19	rs9998591	0.0031
<i>NFATC1/NFAT2</i>	rs8090692	NFATC1/NFAT2	rs177820	0.0062
<i>NFATC1/NFAT2</i>	rs2036892	NFATC1/NFAT2	rs7227107	0.0095
<i>RBAK</i>	rs7805748	TNFSF13B	rs9520835	0.0099
<i>RNF7</i>	rs6769676	ATR	rs13085998	0.0004
<i>RNF7</i>	rs6769676	ATR	rs4273389	0.0001
<i>RNF7</i>	rs6769676	ATR	rs7651071	<.0001
<i>RNF7</i>	rs6769676	TLR10	rs11096955	0.0068
<i>RNF7</i>	rs1980191	TLR10	rs11096957	0.0008
<i>RNF7</i>	rs6769676	TNIP2	rs4690055	0.0042
<i>RNF7</i>	rs6769676	WDR19	rs3733280	0.0049
<i>SOCS6</i>	rs713130	NFATC1/NFAT2	rs7227107	0.0066
<i>SPSB4</i>	rs1108693	HTT	rs2285086	0.0098
<i>SPSB4</i>	rs1108693	TNFSF13B	rs10508198	0.0079
<i>STAT1</i>	rs6751855	NFATC1/NFAT2	rs370989	0.0093
<i>STAT1</i>	rs6751855	NFATC1/NFAT2	rs3894049	0.0018
<i>STAT1</i>	rs2280233	TFDP1	rs4150703	0.0045
<i>STAT1</i>	rs12468579	TLR10	rs11096957	0.0049
<i>STAT1</i>	rs7562024	TNFRSF11A	rs4941125	0.0094
<i>STAT1</i>	rs12468579	TRIAD3	rs13246406	0.0027
<i>STAT4</i>	rs1517352	CARD11/CARMA1	rs1713911	0.003

Gene 1	SNP 1	Gene 2	SNP 2	p-value
<i>STAT4</i>	rs4341967	NFATC1/NFAT2	rs177820	0.0082
<i>STAT4</i>	rs897200	NFATC1/NFAT2	rs4799055	0.0028
<i>STAT4</i>	rs1517352	TLR10	rs11096955	0.0022
<i>STAT4</i>	rs1517352	TLR10	rs11096957	0.0001
<i>STAT4</i>	rs4341967	TLR10	rs7658893	0.006
<i>STAT4</i>	rs11693480	TNFRSF11A	rs7236060	0.0064
<i>STAT4</i>	rs12463658	UBE2K	rs302947	0.001
<i>SUMO1</i>	rs3754931	EMP	rs1680073	0.0018
<i>SUMO1</i>	rs6755690	NFATC1/NFAT2	rs177820	0.0057
<i>SUMO1</i>	rs6755690	TLR1	rs5743565	0.0086
<i>TFDP1</i>	rs12428926	TNFRSF11A	rs4263037	0.0076
<i>TFDP2</i>	rs2163294	HTT	rs6855981	0.0046
<i>TFDP2</i>	rs2163294	ING1	rs4773240	0.0099
<i>TFDP2</i>	rs7642874	EMP	rs12641735	0.0026
<i>TFDP2</i>	rs7642874	NFATC1/NFAT2	rs1667673	0.0036
<i>TFDP2</i>	rs9877536	NFATC1/NFAT2	rs4799055	0.0043
<i>TFDP2</i>	rs7642874	TNFSF13B	rs9514828	0.0077
<i>TFDP2</i>	rs2163294	TNFSF13B	rs9520835	0.0035
<i>TFDP2</i>	rs9877536	TRIAD3	rs2302907	0.0075
<i>TLR1</i>	rs4833095	CARD11/CARMA1	rs10236776	0.0081
<i>TLR1</i>	rs5743565	NFATC1/NFAT2	rs4799055	0.0016
<i>TLR1</i>	rs5743565	SOCS6	rs713130	0.0016
<i>TLR1</i>	rs4833095	TNFRSF11A	rs7239261	0.0044
<i>TLR1</i>	rs4833095	TRIAD3	rs10257204	0.0043
<i>TLR1</i>	rs4833095	TRIAD3	rs2302907	0.0027
<i>TLR1</i>	rs5743565	TRIAD3	rs2302907	0.0028
<i>TLR10</i>	rs10776482	CARD11/CARMA1	rs12538346	0.0037
<i>TLR10</i>	rs10776482	NFATC1/NFAT2	rs12608349	0.0036
<i>TLR10</i>	rs11096955	SOCS6	rs713130	0.0084
<i>TLR10</i>	rs7658893	SOCS6	rs713130	0.003
<i>TLR10</i>	rs11096957	TLR6	rs5743818	0.0027
<i>TLR10</i>	rs11096957	TNFRSF11A	rs12959396	0.0039
<i>TLR10</i>	rs11096957	TNFRSF11A	rs8094884	0.0062
<i>TLR10</i>	rs11096957	TRIAD3	rs2112006	0.0099
<i>TLR10</i>	rs7658893	UBE2K	rs4263408	0.0043
<i>TLR6</i>	rs5743818	CARD11/CARMA1	rs7805748	0.0087
<i>TLR6</i>	rs3821985	NFATC1/NFAT2	rs9962479	0.0019
<i>TLR6</i>	rs5743818	TFDP1	rs12428926	0.0049
<i>TLR6</i>	rs5743810	TRIAD3	rs852417	0.0049
<i>TLR6</i>	rs3821985	UBE2K	rs13122400	0.0081
<i>TNFRSF11A</i>	rs2981007	NFATC1/NFAT2	rs183374	0.0082
<i>TNFSF13B</i>	rs1224163	TNFRSF11A	rs2981007	0.0083
<i>TRIAD3</i>	rs852394	ING1	rs4773240	0.0016
<i>TRIAD3</i>	rs852374	NFATC1/NFAT2	rs177820	0.01
<i>TRIAD3</i>	rs13239194	TNFRSF11A	rs2957125	0.0081
<i>TRIAD3</i>	rs13239194	TNFSF13B	rs10508198	0.0082
<i>TRIAD3</i>	rs13239194	TRIAD3	rs10257204	0.0052

Gene 1	SNP 1	Gene 2	SNP 2	p-value
<i>TRIAD3</i>	rs852417	TRIAD3	rs2302907	0.001
<i>TRIAD3</i>	rs852417	TRIAD3	rs852394	0.0036
<i>UBE2K</i>	rs4263408	TFDP1	rs4150703	0.0049
<i>UBE2K</i>	rs302947	UBE2K	rs4263408	0.0091
<i>WDR19</i>	rs9997015	NFATC1/NFAT2	rs1667673	0.0083
<i>WDR19</i>	rs1057807	NFATC1/NFAT2	rs7227107	0.0069
<i>WDR19</i>	rs9997015	RBAK	rs7805748	0.0092
<i>WDR19</i>	rs9997015	TNFRSF11A	rs4263037	0.0074
<i>WDR19</i>	rs12648082	TNFSF13B	rs1224163	0.0085
<i>WDR19</i>	rs9997015	TNFSF13B	rs9514828	0.0021
<i>WDR19</i>	rs12648082	TRIAD3	rs2112006	0.0061
<i>WDR19</i>	rs1057807	UBE2K	rs302947	0.0008
<i>ZAK</i>	rs12618933	ATF2/CREB2	rs10930693	0.0019
<i>ZAK</i>	rs1837470	CERKL	rs1047307	0.0064
<i>ZAK</i>	rs13032010	CREB1	rs10932201	0.0007
<i>ZAK</i>	rs3769192	GULP1	rs10931359	0.0014
<i>ZAK</i>	rs17302977	GULP1	rs1354905	0.009
<i>ZAK</i>	rs12618933	HTT	rs362336	0.0093
<i>ZAK</i>	rs4972533	NFATC1/NFAT2	rs12608349	0.0012
<i>ZAK</i>	rs13032010	NFATC1/NFAT2	rs177820	0.0065
<i>ZAK</i>	rs12618933	NFATC1/NFAT2	rs7227107	0.0098
<i>ZAK</i>	rs989531	RBAK	rs7805748	0.0069
<i>ZAK</i>	rs11686011	RNF7	rs6769676	0.0075
<i>ZAK</i>	rs12618933	STAT4	rs11685878	0.0071
<i>ZAK</i>	rs4972533	STAT4	rs2356350	0.0035
<i>ZAK</i>	rs6759787	SUMO1	rs6717044	0.0025
<i>ZAK</i>	rs13032010	TLR10	rs7658893	0.0098
<i>ZAK</i>	rs12618933	TLR6	rs5743818	0.0065
<i>ZAK</i>	rs6759787	TNFRSF11A	rs7239261	0.0042
<i>ZAK</i>	rs12618933	UBE2K	rs4263408	0.0031

Table B5. Gene-gene interactions of clubfoot deletion region apoptotic genes p<0.01 in Hispanics.

Gene 1	SNP 1	Gene 2	SNP 2	p-value
<i>ATF2/CREB2</i>	rs212352	HTT	rs6446723	0.0089
<i>ATF2/CREB2</i>	rs2698545	NFATC1/NFAT2	rs8097537	0.0072
<i>ATR</i>	rs13085998	CARD11/CARMA1	rs1713911	0.0011
<i>ATR</i>	rs13085998	ING1	rs6492308	0.0095
<i>ATR</i>	rs13085998	TNFRSF11A	rs7236060	0.0053
<i>ATR</i>	rs3922730	TNIP2	rs4690055	0.0028
<i>ATR</i>	rs4273389	NFATC1/NFAT2	rs1660139	0.0079
<i>ATR</i>	rs6440085	NFATC1/NFAT2	rs4799055	0.0077
<i>ATR</i>	rs7651071	CARD11/CARMA1	rs1713911	0.0065
<i>ATR</i>	rs7651071	WDR19	rs11096987	0.0013
<i>ATR</i>	rs9816736	WDR19	rs11096987	0.0033
<i>CARD11/CARMA1</i>	rs10236776	TNFRSF11A	rs2981007	0.009
<i>CARD11/CARMA1</i>	rs10236776	TRIAD3	rs2302907	0.0097
<i>CARD11/CARMA1</i>	rs11982651	ING1	rs1441043	0.0057
<i>CARD11/CARMA1</i>	rs1713911	ING1	rs1441043	<.0001
<i>CARD11/CARMA1</i>	rs1713911	TNFRSF11A	rs8089829	0.0021
<i>CARD11/CARMA1</i>	rs4722276	TNFRSF11A	rs8094884	0.0047
<i>CARD11/CARMA1</i>	rs4722276	TRIAD3	rs3779092	0.0071
<i>CARD11/CARMA1</i>	rs4722356	TNFSF13B	rs10508198	0.0076
<i>CERKL</i>	rs10445770	TNIP2	rs4690060	0.002
<i>CERKL</i>	rs10445770	TRIAD3	rs3823681	0.0043
<i>CERKL</i>	rs10445770	UBE2K	rs12644528	0.0099
<i>CERKL</i>	rs1047307	CARD11/CARMA1	rs11982651	0.0018
<i>CERKL</i>	rs1047307	GULP1	rs7593546	0.0087
<i>CERKL</i>	rs1047307	NFATC1/NFAT2	rs177820	0.0009
<i>CERKL</i>	rs11680383	TRIAD3	rs13247447	0.0088
<i>CERKL</i>	rs1866888	HTT	rs362331	0.0007
<i>CERKL</i>	rs1992394	NFATC1/NFAT2	rs177820	0.0062
<i>CERKL</i>	rs1992394	SUMO1	rs3754931	0.0044
<i>CERKL</i>	rs1992394	TLR10	rs10776482	0.0073
<i>CERKL</i>	rs1992394	TNFRSF11A	rs2981007	0.0044
<i>CERKL</i>	rs1992394	TNFSF13B	rs9520835	0.01
<i>CERKL</i>	rs895901	HTT	rs6446723	0.0066
<i>CERKL</i>	rs895901	TLR10	rs11096957	0.0025
<i>CERKL</i>	rs895901	TRIAD3	rs13247447	0.0065
<i>CERKL</i>	rs935087	GULP1	rs12474692	0.0021
<i>CERKL</i>	rs935087	TLR1	rs4833095	0.0034
<i>CERKL</i>	rs935087	TRIAD3	rs6971918	0.0075
<i>CREB1</i>	rs10932201	HTT	rs10015979	0.01
<i>CREB1</i>	rs2253206	TRIAD3	rs10257204	0.0098
<i>GULP1</i>	rs10931346	NFATC1/NFAT2	rs7227107	0.0059
<i>GULP1</i>	rs10931346	RBAK	rs10238244	0.0014
<i>GULP1</i>	rs10931359	ATR	rs9816736	0.0076
<i>GULP1</i>	rs11685321	STAT1	rs13395505	0.0042

Gene 1	SNP 1	Gene 2	SNP 2	p-value
<i>GULP1</i>	rs12474692	ATR	rs13085998	0.007
<i>GULP1</i>	rs12474692	RBAK	rs7778444	0.0003
<i>GULP1</i>	rs12474692	SOCS6	rs7230661	0.0015
<i>GULP1</i>	rs13034731	ATR	rs6440085	0.0058
<i>GULP1</i>	rs13034731	WDR19	rs12648082	0.0066
<i>GULP1</i>	rs13034731	WDR19	rs1451821	0.0054
<i>GULP1</i>	rs13034731	WDR19	rs3733280	0.0025
<i>GULP1</i>	rs4396679	CREB1	rs10932201	0.0042
<i>GULP1</i>	rs4396679	RBAK	rs10238244	0.0051
<i>GULP1</i>	rs6724428	RBAK	rs7778444	0.0066
<i>GULP1</i>	rs7586390	ATR	rs13085998	0.01
<i>GULP1</i>	rs7586390	RBAK	rs7778444	0.0006
<i>HTT</i>	rs10015979	HTT	rs2269478	0.0069
<i>HTT</i>	rs10015979	NFATC1/NFAT2	rs2036892	0.0083
<i>HTT</i>	rs10015979	WDR19	rs12648082	0.0045
<i>HTT</i>	rs362331	CARD11/CARMA1	rs4722356	0.0056
<i>HTT</i>	rs362336	NFATC1/NFAT2	rs1667673	0.007
<i>HTT</i>	rs362336	NFATC1/NFAT2	rs4799055	0.0014
<i>HTT</i>	rs362336	TNFRSF11A	rs2981007	0.0045
<i>HTT</i>	rs362336	TNFRSF11A	rs8089829	0.005
<i>HTT</i>	rs362336	TNFSF13B	rs8181791	0.005
<i>HTT</i>	rs362336	TNFSF13B	rs9514828	0.0057
<i>HTT</i>	rs363092	NFATC1/NFAT2	rs7227107	0.0065
<i>HTT</i>	rs363092	TRIAD3	rs13247447	0.007
<i>HTT</i>	rs4690074	TLR10	rs11096957	0.0054
<i>HTT</i>	rs762855	NFATC1/NFAT2	rs177820	0.0011
<i>ING1</i>	rs4773240	NFATC1/NFAT2	rs7227107	0.0056
<i>EMP</i>	rs11727167	HTT	rs363092	0.008
<i>EMP</i>	rs12641735	SOCS6	rs713130	0.0095
<i>EMP</i>	rs12647145	NFATC1/NFAT2	rs3894049	0.0056
<i>EMP</i>	rs1680073	TLR10	rs7658893	0.0054
<i>RBAK</i>	rs7778444	TFDP1	rs4150703	0.0065
<i>RBAK</i>	rs7778444	TNFRSF11A	rs4263037	0.0028
<i>RBAK</i>	rs7778444	TNFRSF11A	rs7236060	0.0018
<i>RBAK</i>	rs7778444	TNFRSF11A	rs8089829	0.0009
<i>RBAK</i>	rs7778444	TNFSF13B	rs9514828	0.0008
<i>RBAK</i>	rs7805748	NFATC1/NFAT2	rs177820	0.0082
<i>RBAK</i>	rs7805748	TFDP1	rs7325214	0.002
<i>RBAK</i>	rs7805748	TFDP1	rs9577595	0.003
<i>RNF7</i>	rs1980191	HTT	rs363092	0.0011
<i>RNF7</i>	rs6769676	UBE2K	rs305827	0.0036
<i>RNF7</i>	rs6776205	CARD11/CARMA1	rs11982651	0.0028
<i>RNF7</i>	rs6776205	EMP	rs7664474	0.0029
<i>RNF7</i>	rs6776205	TNFRSF11A	rs12959396	0.0018
<i>RNF7</i>	rs6776205	TRIAD3	rs13247447	0.0005
<i>RNF7</i>	rs6776205	TRIAD3	rs852374	0.0043
<i>SOC6</i>	rs2053420	NFATC1/NFAT2	rs1667673	0.0016

Gene 1	SNP 1	Gene 2	SNP 2	p-value
<i>STAT1</i>	rs12468579	STAT4	rs925847	0.0008
<i>STAT1</i>	rs13395505	CARD11/CARMA1	rs4722276	0.0031
<i>STAT1</i>	rs13395505	TNFRSF11A	rs8089829	0.0087
<i>STAT1</i>	rs2280233	EMP	rs12641735	0.0017
<i>STAT1</i>	rs2280233	EMP	rs12647145	0.0095
<i>STAT1</i>	rs2280233	UBE2K	rs302947	0.0081
<i>STAT1</i>	rs6751855	TRIAD3	rs1468996	0.0062
<i>STAT1</i>	rs7562024	HTT	rs363092	0.0096
<i>STAT1</i>	rs7562024	TNFRSF11A	rs2981007	0.0055
<i>STAT4</i>	rs11685878	CARD11/CARMA1	rs4722356	0.01
<i>STAT4</i>	rs1517352	SOCS6	rs7230661	0.0056
<i>STAT4</i>	rs16833215	HTT	rs363096	0.0031
<i>STAT4</i>	rs16833215	SUMO1	rs3754931	0.0049
<i>STAT4</i>	rs16833215	TNIP2	rs9683949	0.0095
<i>STAT4</i>	rs16833215	TRIAD3	rs11771172	0.0018
<i>STAT4</i>	rs16833215	TRIAD3	rs13246406	0.001
<i>STAT4</i>	rs16833249	RNF7	rs6776205	0.0095
<i>STAT4</i>	rs4853543	NFATC1/NFAT2	rs12608349	0.0024
<i>STAT4</i>	rs4853543	STAT4	rs6738544	0.0089
<i>STAT4</i>	rs6738544	ATR	rs13085998	0.0066
<i>STAT4</i>	rs6738544	TRIAD3	rs13246406	0.005
<i>STAT4</i>	rs7572482	CARD11/CARMA1	rs10236776	0.0039
<i>STAT4</i>	rs7572482	UBE2K	rs3912392	0.002
<i>STAT4</i>	rs7574909	NFATC1/NFAT2	rs8090692	0.0033
<i>STAT4</i>	rs7574909	RNF7	rs6769676	0.0035
<i>STAT4</i>	rs925847	EMP	rs11727167	0.0052
<i>STAT4</i>	rs925847	EMP	rs12642410	0.0012
<i>STAT4</i>	rs925847	EMP	rs1316393	0.0057
<i>STAT4</i>	rs925847	EMP	rs7673398	0.0035
<i>STAT4</i>	rs925847	RNF7	rs1980191	0.0014
<i>STAT4</i>	rs925847	STAT4	rs1517352	0.0032
<i>STAT4</i>	rs925847	STAT4	rs4853543	0.0034
<i>STAT4</i>	rs925847	TNFRSF11A	rs12959396	0.0091
<i>STAT4</i>	rs925847	TNFRSF11A	rs8094884	0.0003
<i>STAT4</i>	rs925847	UBE2K	rs13122400	0.0035
<i>SUMO1</i>	rs4675272	SUMO1	rs3754931	0.0012
<i>SUMO1</i>	rs4675272	TNFSF13B	rs9520835	0.0002
<i>SUMO1</i>	rs4675272	TRIAD3	rs11771172	0.0012
<i>SUMO1</i>	rs6717044	HTT	rs2285086	0.0055
<i>SUMO1</i>	rs6717044	RBAK	rs7778444	0.0012
<i>TFDP1</i>	rs7325214	NFATC1/NFAT2	rs7227107	0.0007
<i>TFDP1</i>	rs7325214	TNFRSF11A	rs4263037	0.0051
<i>TFDP1</i>	rs7325214	TNFRSF11A	rs8089829	0.0028
<i>TFDP1</i>	rs9577595	NFATC1/NFAT2	rs4799055	0.0097
<i>TFDP1</i>	rs9577595	NFATC1/NFAT2	rs7227107	0.0027
<i>TFDP1</i>	rs9577595	TNFRSF11A	rs8089829	0.0077
<i>TLR1</i>	rs4833095	TNFSF13B	rs1224163	0.0052

Gene 1	SNP 1	Gene 2	SNP 2	p-value
<i>TLR1</i>	rs4833095	TRIAD3	rs10257204	0.005
<i>TLR10</i>	rs10776482	CARD11/CARMA1	rs10236776	0.0049
<i>TLR10</i>	rs11096955	CARD11/CARMA1	rs10236776	0.0055
<i>TLR6</i>	rs5743818	UBE2K	rs10440307	0.0078
<i>TNFRSF11A</i>	rs4263037	TNFRSF11A	rs8089829	0.002
<i>TNFRSF11A</i>	rs7236060	NFATC1/NFAT2	rs177820	0.0051
<i>TNFRSF11A</i>	rs9646629	NFATC1/NFAT2	rs4799055	0.0086
<i>TNFSF13B</i>	rs9514828	NFATC1/NFAT2	rs2290154	0.009
<i>TNFSF13B</i>	rs9520835	TNFRSF11A	rs4941125	0.0069
<i>TNIP2</i>	rs4690060	NFATC1/NFAT2	rs8090692	0.0058
<i>TNIP2</i>	rs4690060	TRIAD3	rs2017620	0.0019
<i>TNIP2</i>	rs4690060	TRIAD3	rs6971918	0.0063
<i>TNIP2</i>	rs9683949	TRIAD3	rs13246406	0.0053
<i>TRIAD3</i>	rs2017620	TNFRSF11A	rs7239261	0.0061
<i>TRIAD3</i>	rs3779092	NFATC1/NFAT2	rs8090692	0.0029
<i>TRIAD3</i>	rs3823681	TNFRSF11A	rs7239261	0.0057
<i>TRIAD3</i>	rs6967635	TNFRSF11A	rs7239261	0.0062
<i>TRIAD3</i>	rs6971918	TNFRSF11A	rs7239261	0.0018
<i>TRL6</i>	rs3821985	UBE2K	rs305827	0.0005
<i>UBE2K</i>	rs12644528	TRIAD3	rs2017620	0.0046
<i>UBE2K</i>	rs305827	NFATC1/NFAT2	rs8090692	0.0047
<i>UBE2K</i>	rs305827	TRIAD3	rs6967635	0.0066
<i>UBE2K</i>	rs305827	TRIAD3	rs6971918	0.0055
<i>UBE2K</i>	rs3912392	CARD11/CARMA1	rs11982651	0.0069
<i>UBE2K</i>	rs3912392	NFATC1/NFAT2	rs8090692	0.0052
<i>WDR19</i>	rs12648082	CARD11/CARMA1	rs1713911	0.0033
<i>WDR19</i>	rs1451821	CARD11/CARMA1	rs1713911	0.0065
<i>WDR19</i>	rs3733280	RBAK	rs7805748	0.0082
<i>WDR19</i>	rs3733280	TNFRSF11A	rs2981007	0.0032
<i>WDR19</i>	rs6815686	WDR19	rs3733280	0.0072
<i>ZAK</i>	rs11685001	CERKL	rs10445770	0.0029
<i>ZAK</i>	rs11685001	CREB1	rs2254137	0.0098
<i>ZAK</i>	rs11685001	TRIAD3	rs3779092	0.0057
<i>ZAK</i>	rs11686011	CERKL	rs10445770	0.006
<i>ZAK</i>	rs11686011	STAT4	rs4341967	0.0006
<i>ZAK</i>	rs13032010	CARD11/CARMA1	rs10236776	0.0063
<i>ZAK</i>	rs13032010	CERKL	rs10445770	0.0061
<i>ZAK</i>	rs13032010	SOCS6	rs7230661	0.0052
<i>ZAK</i>	rs1837470	RNF7	rs6769676	0.005
<i>ZAK</i>	rs2028382	CERKL	rs10445770	0.0023
<i>ZAK</i>	rs2028382	TFDP1	rs9577595	0.0019
<i>ZAK</i>	rs3769192	CERKL	rs10445770	0.0088
<i>ZAK</i>	rs4972533	CERKL	rs10445770	0.0013
<i>ZAK</i>	rs4972533	TRIAD3	rs3779092	0.0003
<i>ZAK</i>	rs4972533	TRL6	rs3821985	0.0096
<i>ZAK</i>	rs6433395	STAT4	rs16833249	0.0078
<i>ZAK</i>	rs6433395	TNFRSF11A	rs7239261	0.0097

Gene 1	SNP 1	Gene 2	SNP 2	p-value
<i>ZAK</i>	rs6759787	NFATC1/NFAT2	rs177820	0.0098
<i>ZAK</i>	rs989531	HTT	rs362336	0.0024
<i>ZAK</i>	rs989531	UBE2K	rs3912392	0.0097

Appendix C: HOXA and HOXD Supplemental Tables

Supplemental Table I. Linkage disequilibrium (D') for *HOXD* (A) and *HOXA* (B) for nonHispanic white population^{a,b}

A.

		rs6749771	rs1446575	rs1318778	rs1542180	rs1867863	rs2113563	rs2592394	rs847146	rs741610	rs711812	rs6758117
		SNP1	SNP2	SNP3	SNP4	SNP5	SNP6	SNP7	SNP8	SNP9	SNP10	SNP11
rs6749771	SNP1		0.789	0.613	0.795	0.715	0.39	0.401	0.094	0.273	0.193	0.2
rs1446575	SNP2	0.712		0.973	0.99	0.899	0.483	0.484	0.163	0.32	0.286	0.332
rs1318778	SNP3	0.384	0.935		1	0.845	0.514	0.45	0.236	0.582	0.492	0.013
rs1542180	SNP4	0.708	0.986	1		0.902	0.513	0.52	0.169	0.334	0.255	0.35
rs1867863	SNP5	0.632	0.926	0.824	0.928		0.759	0.471	0.158	0.32	0.244	0.413
rs2113563	SNP6	0.13	0.39	0.479	0.418	0.814		0.825	0.387	0.108	0.042	0.335
rs2592394	SNP7	0.22	0.522	0.499	0.572	0.679	0.856		0.008	0.488	0.305	0.295
rs847146	SNP8	0.153	0.242	0.33	0.254	0.235	0.387	0.182		0.762	0.72	0.267
rs741610	SNP9	0.226	0.316	0.608	0.324	0.242	0.02	0.314	0.74		0.988	0.425
rs711812	SNP10	0.196	0.288	0.514	0.282	0.218	0.011	0.289	0.695	0.985		0.362
rs6758117	SNP11	0.234	0.438	0.397	0.422	0.417	0.406	0.461	0.331	0.31	0.322	

^aD' of affecteds above diagonal; D' of normals below diagonal.

^bD'>0.8 shown in dark orange; 0.6<D'<0.8 shown in medium orange; 0.3<D'<0.6 shown in pale yellow

B.

		rs2462907	rs6668	rs2428431	rs3757640	rs3801776	rs3779456	rs1859164	rs6968828	rs3807598
		SNP12	SNP13	SNP14	SNP15	SNP16	SNP17	SNP18	SNP19	SNP20
rs2462907	SNP12		0.773	0.749	0.156	0.016	0.257	0.207	0.068	0.126
rs6668	SNP13	0.767		0.948	0.638	0.601	0.195	0.232	0.04	0.151
rs2428431	SNP14	0.747	0.966		0.698	0.601	0.181	0.226	0.018	0.119
rs3757640	SNP15	0.07	0.697	0.701		0.56	0.055	0.545	0.318	0.324
rs3801776	SNP16	0.06	0.57	0.555	0.557		0.027	0.451	0.313	0.265
rs3779456	SNP17	0.245	0.198	0.178	0.063	0.211		0.975	0.498	0.701
rs1859164	SNP18	0.226	0.143	0.134	0.559	0.651	0.992		0.779	0.728
rs6968828	SNP19	0.155	0.074	0.045	0.353	0.476	0.515	0.733		0.941
rs3807598	SNP20	0.164	0.163	0.117	0.363	0.477	0.721	0.77	0.966	

^aD' of affecteds above diagonal; D' of normals below diagonal.

^bD' > 0.8 shown in dark orange; 0.6 < D' < 0.8 shown in medium orange; 0.3 < D' < 0.6 shown in pale yellow

Supplemental Table III. Linkage disequilibrium (D') for *HOXD* (A) and *HOXA* (B) for Hispanic population^{a,b}

A.

		rs6749771	rs1446575	rs1318778	rs1542180	rs1867863	rs2113563	rs2592394	rs847146	rs741610	rs711812	rs6758117
		SNP1	SNP2	SNP3	SNP4	SNP5	SNP6	SNP7	SNP8	SNP9	SNP10	SNP11
rs6749771	SNP1		0.778	0.671	0.775	0.786	0.303	0.478	0.231	0.403	0.422	0.343
rs1446575	SNP2	0.772		1	1	0.951	0.351	0.726	0.231	0.472	0.414	0.477
rs1318778	SNP3	0.615	0.964		1	0.97	0.631	0.612	0.366	0.586	0.503	0.324
rs1542180	SNP4	0.762	0.978	1		0.951	0.348	0.725	0.228	0.469	0.41	0.484
rs1867863	SNP5	0.791	0.955	0.956	0.939		0.819	0.707	0.13	0.24	0.241	0.403
rs2113563	SNP6	0.216	0.302	0.674	0.322	0.766		0.93	0.144	0.111	0.106	0.34
rs2592394	SNP7	0.37	0.588	0.676	0.655	0.596	0.928		0.235	0.412	0.249	0.361
rs847146	SNP8	0.214	0.356	0.466	0.353	0.241	0.107	0.159		0.739	0.685	0.29
rs741610	SNP9	0.312	0.401	0.519	0.436	0.271	0.036	0.447	0.713		0.974	0.046
rs711812	SNP10	0.352	0.415	0.556	0.433	0.28	0.053	0.388	0.642	0.969		0.059
rs6758117	SNP11	0.408	0.499	0.571	0.528	0.425	0.365	0.378	0.546	0.089	0.08	

^aD' of affecteds above diagonal; D' of normals below diagonal.

^bD'>0.8 shown in dark orange; 0.6<D'<0.8 shown in medium orange; 0.3<D'<0.6 shown in pale yellow

B.

		rs2462907	rs6668	rs2428431	rs3757640	rs3801776	rs3779456	rs1859164	rs6968828	rs3807598
		SNP12	SNP13	SNP14	SNP15	SNP16	SNP17	SNP18	SNP19	SNP20
rs2462907	SNP12		0.696	0.706	0.217	0.254	0.172	0.254	0.086	0.082
rs6668	SNP13	0.707		0.982	0.611	0.453	0.276	0.257	0.052	0.044
rs2428431	SNP14	0.724	0.975		0.626	0.439	0.294	0.282	0.089	0.022
rs3757640	SNP15	0.203	0.415	0.448		0.605	0.158	0.403	0.189	0.14
rs3801776	SNP16	0.406	0.574	0.6	0.672		0.506	0.715	0.187	0.676
rs3779456	SNP17	0.031	0.088	0.14	0.181	0.56		0.967	0.072	0.779
rs1859164	SNP18	0.095	0.012	0.068	0.405	0.766	0.969		0.26	0.755
rs6968828	SNP19	0.209	0.126	0.126	0.302	0.173	0.317	0.493		0.934
rs3807598	SNP20	0.032	0.158	0.137	0.148	0.632	0.812	0.731	0.971	

^aD' of affecteds above diagonal; D' of normals below diagonal.

^bD' > 0.8 shown in dark orange; 0.6 < D' < 0.8 shown in medium orange; 0.3 < D' < 0.6 shown in pale yellow

Supplemental Table V. Results of APL analysis of *HOXA* haplotypes in discovery population^{a,b,c}

		rs2462907	rs6668	rs2428431	rs3757640	rs3801776	rs3779456	rs1859164	rs6968828	rs3807598
SNP	Pos.	SNP12	SNP13	SNP14	SNP15	SNP16	SNP17	SNP18	SNP19	SNP20
rs2462907	SNP12		0.878	0.527	0.493	0.035	0.507	0.362	0.604	0.148
rs6668	SNP13	0.119		0.206	0.351	0.022	0.416	0.529	0.803	0.165
rs2428431	SNP14	0.051	0.065		0.350	0.020	0.672	0.510	0.597	0.264
rs3757640	SNP15	0.016	0.037	0.009		0.028	0.191	0.380	0.466	0.151
rs3801776	SNP16	0.006	0.050	0.019	0.105		0.018	0.004	0.017	0.015
rs3779456	SNP17	0.049	0.066	0.058	0.804	0.426		0.451	0.509	0.235
rs1859164	SNP18	0.029	0.017	0.035	0.675	0.255	0.405		0.848	0.364
rs6968828	SNP19	0.218	0.031	0.006	0.629	0.383	0.422	0.730		0.495
rs3807598	SNP20	0.165	0.156	0.103	0.830	0.348	0.749	0.696	0.580	

^aHispanic p values shown below diagonal, nonHispanic white above the diagonal.

^bp<0.01 shown in red and p<0.05 shown in blue, respectively.

^cp-values uncorrected for multiple testing

Supplemental Table VI. Results of APL analysis of *HOXD* haplotypes in discovery population^{a,b,c}

		rs6749771	rs1446575	rs1318778	rs1542180	rs1867863	rs2113563	rs2592394	rs847146	rs741610	rs711812	rs6758117
SNP	Pos.	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6	SNP7	SNP8	SNP9	SNP10	SNP11
rs6749771	SNP1		0.007	0.037	0.019	0.018	0.007	0.006	0.010	0.100	0.076	0.101
rs1446575	SNP2	0.761		0.090	0.136	0.224	0.111	0.071	0.101	0.147	0.104	0.086
rs1318778	SNP3	0.416	0.543		0.138	0.483	0.426	0.606	0.660	0.259	0.790	0.197
rs1542180	SNP4	0.699	0.068	0.643		0.256	0.214	0.182	0.151	0.375	0.201	0.267
rs1867863	SNP5	0.903	0.829	0.621	0.527		0.366	0.374	0.407	0.712	0.572	0.438
rs2113563	SNP6	0.862	0.662	0.196	0.604	0.314		0.804	0.107	0.347	0.113	0.646
rs2592394	SNP7	0.312	0.188	0.038	0.204	0.213	0.671		0.491	0.909	0.331	0.643
rs847146	SNP8	0.992	0.710	0.300	0.544	0.653	0.883	0.626		0.520	0.594	0.560
rs741610	SNP9	0.888	0.766	0.557	0.849	0.724	0.931	0.586	0.709		0.282	0.792
rs711812	SNP10	0.915	0.752	0.198	0.838	0.811	0.806	0.499	0.653	0.508		0.831
rs6758117	SNP11	0.872	0.875	0.753	0.795	0.731	0.853	0.341	0.015	0.564	0.606	

^aHispanic shown below diagonal, nonHispanic white above the diagonal.

^bp<0.01 shown in red and p<0.05 shown in blue, respectively.

^cp-values uncorrected for multiple testing

Supplemental Table IX. Results of GEE Analysis for gene-gene interactions between *HOXA* and *HOXD* and *IGFBP3* in nonHispanic whites^{a,b}

Gene1	SNP1	Gene2	SNP2	p-value
<i>HOXD</i>	rs1446575 (SNP2)	<i>IGFBP3</i>	rs2453839 (SNP30)	0.022
	rs1318778 (SNP3)		rs3793345 (SNP26)	0.034
	rs1318778 (SNP3)		rs2471551 (SNP27)	0.019
	rs1542180 (SNP4)		rs2453839 (SNP30)	0.018
	rs1867863 (SNP5)		rs2453839 (SNP30)	0.013
	rs2113563 (SNP6)		rs3793345 (SNP26)	0.008
	rs2113563 (SNP6)		rs2471551 (SNP27)	0.007
	rs2592394 (SNP7)		rs2132571 (SNP21)	0.024
	rs2592394 (SNP7)		rs2854744 (SNP23)	0.047
	rs2592394 (SNP7)		rs2854746 (SNP24)	0.012
	rs2592394 (SNP7)		rs3793345 (SNP26)	0.003
	rs2592394 (SNP7)		rs2471551 (SNP27)	0.001
	rs2592394 (SNP7)		rs3110697 (SNP28)	0.044
	<i>HOXA</i>		rs3757640 (SNP15)	rs13223993 (SNP32)
rs3801776 (SNP16)		rs2132572 (SNP22)	0.025	
rs3801776 (SNP16)		rs13223993 (SNP32)	<0.001	
rs1859164 (SNP18)		rs2854744 (SNP23)	0.027	
rs6968828 (SNP19)		rs2854744 (SNP23)	0.019	
rs6968828 (SNP19)		rs2854747 (SNP25)	0.003	
rs6968828 (SNP19)		rs3110697 (SNP28)	0.001	
rs3807598 (SNP20)		rs2854747 (SNP25)	0.033	
rs3807598 (SNP20)		rs3110697 (SNP28)	0.011	

^ap<0.05 shown and p<0.01 in bold

^bp-values uncorrected for multiple testing

Supplemental Table X. Results of GEE Analysis for gene-gene interactions between *HOXA* and *HOXD* and *IGFBP3* in Hispanic population^{a,b}

Gene1	SNP1	Gene2	SNP2	p-value
<i>HOXD</i>	rs1318778 (SNP3)	<i>IGFBP3</i>	rs13223993 (SNP32)	0.015
	rs847146 (SNP8)		rs13223993 (SNP32)	0.022
	rs741610 (SNP9)		rs13223993 (SNP32)	0.002
	rs711812 (SNP10)		rs13223993 (SNP32)	0.003
	rs6758117 (SNP11)		rs6670 (SNP31)	0.025
<i>HOXA</i>	rs3801776 (SNP16)		rs2132571 (SNP21)	0.018
	rs3779456 (SNP17)		rs2132571 (SNP21)	0.032
	rs1859164 (SNP18)		rs2132571 (SNP21)	0.033

^ap<0.05 shown, p<0.01 in bold

^bp-values uncorrected for multiple testing

Supplemental Table XI. Results of GEE analysis for gene-gene interactions between *HOXA* and *HOXD* and *IGFBP3* in validation population^{a,b}

Gene 1	SNP1	Gene2	SNP2	p-value
<i>HOXD</i>	rs6749771 (SNP1)	<i>IGFBP3</i>	rs3110697 (SNP28)	0.016
	rs1542180 (SNP4)		rs2132572 (SNP22)	0.035
	rs1542180 (SNP4)		rs2854747 (SNP25)	0.002
	rs1542180 (SNP4)		rs3793345 (SNP26)	0.035
	rs1542180 (SNP4)		rs2471551 (SNP27)	0.044
	rs1542180 (SNP4)		rs3110697 (SNP28)	0.0003
	rs1542180 (SNP4)		rs2453839 (SNP30)	0.002
	rs1867863 (SNP5)		rs2854747 (SNP25)	0.031
	rs1867863 (SNP5)		rs2453839 (SNP30)	0.011
	rs2592394 (SNP7)		rs2132572 (SNP22)	0.033
	rs2592394 (SNP7)		rs2854744 (SNP23)	0.037
	rs2592394 (SNP7)		rs2854747 (SNP25)	0.0006
	rs2592394 (SNP7)		rs3793345 (SNP26)	0.021
	rs2592394 (SNP7)		rs2471551 (SNP27)	0.026
	rs2592394 (SNP7)		rs3110697 (SNP28)	<0.0001
	rs2592394 (SNP7)		rs2453839 (SNP30)	0.004
	rs847146 (SNP8)		rs2132572 (SNP22)	0.034
	rs847146 (SNP8)		rs2854747 (SNP25)	0.004
	rs847146 (SNP8)		rs3793345 (SNP26)	0.038
	rs847146 (SNP8)		rs2471551 (SNP27)	0.044
rs847146 (SNP8)	rs3110697 (SNP28)	0.001		
<i>HOXA</i>	rs2462907 (SNP12)	rs3793345 (SNP26)	0.016	
	rs6968828 (SNP19)	rs2471551 (SNP27)	0.007	
		rs6670 (SNP31)	0.016	

^ap<0.05 shown and p<0.01 in bold

^bp-values uncorrected for multiple testing

Supplemental Table XII. Results of GEE Analysis for gene-gene interactions for *HOX*, *IGFBP3* and mitochondrial mediated apoptotic variants in nonHispanic white population^{a,b}

Gene1	SNP1	Gene2	SNP2	p-value
<i>Apaf1</i>	rs2288729	HOXD	rs711812 (SNP10)	0.047
<i>Bid</i>	rs3788284		rs2113563 (SNP6)	0.045
<i>Bid</i>	rs181405		rs847146 (SNP8)	0.026
<i>Bid</i>	rs8919		rs6758117 (SNP11)	0.007
<i>Bcl2</i>	rs1801018		rs6749771 (SNP1)	0.049
<i>Bcl2</i>	rs1809319		rs1867863 (SNP5)	0.025
<i>Bcl2</i>	rs1801018		rs1867863 (SNP5)	0.029
<i>Casp3</i>	rs1049216		rs6749771 (SNP1)	0.002
<i>Casp3</i>	rs1405937		rs6749771 (SNP1)	0.005
<i>Casp3</i>	rs1405944		rs6749771 (SNP1)	0.011
<i>Casp3</i>	rs2720378		rs6749771 (SNP1)	0.014
<i>Casp3</i>	rs4647602		rs6749771 (SNP1)	0.016
<i>Casp3</i>	rs2696057		rs6749771 (SNP1)	0.037
<i>Casp3</i>	rs1049253		rs1446575 (SNP2)	0.031
<i>Casp3</i>	rs1049253		rs1542180 (SNP4)	0.049
<i>Casp3</i>	rs1049216		rs1867863 (SNP5)	0.023
<i>Casp3</i>	rs1049216		rs2113563 (SNP6)	0.018
<i>Casp3</i>	rs1049253		rs2592394 (SNP7)	0.001
<i>Casp3</i>	rs1049216		rs2592394 (SNP7)	0.008
<i>Casp3</i>	rs1405944		rs741610 (SNP9)	0.043
<i>Casp3</i>	rs1049253		rs6758117 (SNP11)	0.035
<i>Casp3</i>	rs2720378		rs6758117 (SNP11)	0.040
<i>Casp9</i>	rs4233533		rs741610 (SNP9)	0.017
<i>Casp9</i>	rs2042370		rs741610 (SNP9)	0.044
<i>Casp10</i>	rs3900115		rs6749771 (SNP1)	0.044
<i>Apaf1</i>	rs7310804		HOXA	rs3779456 (SNP17)
<i>Apaf1</i>	rs7310804	rs1859164 (SNP18)		0.043
<i>Bid</i>	rs3788284	rs2462907 (SNP12)		0.029
<i>Bid</i>	rs5747351	rs2428431 (SNP14)		0.029
<i>Bid</i>	rs8190315	rs3757640 (SNP15)		0.023
<i>Bid</i>	rs8919	rs3801776 (SNP16)		0.016
<i>Bid</i>	rs8919	rs1859164 (SNP18)		0.036
<i>Bid</i>	rs181405	rs3779456 (SNP17)		0.001
<i>Bid</i>	rs181405	rs1859164 (SNP18)		0.005
<i>Casp3</i>	rs1049216	rs6668 (SNP13)		0.014

<i>Casp3</i>	rs1405944	rs6668 (SNP13)	0.010
<i>Casp3</i>	rs2720378	rs6668 (SNP13)	0.004
<i>Casp3</i>	rs2720378	rs2428431 (SNP14)	0.039
<i>Casp3</i>	rs1405937	rs3801776 (SNP16)	0.048
<i>Casp3</i>	rs2720378	rs3779456 (SNP17)	0.033
<i>Casp3</i>	rs1049216	rs1859164 (SNP18)	0.004
<i>Casp3</i>	rs2720378	rs1859164 (SNP18)	0.004
<i>Casp3</i>	rs1405944	rs1859164 (SNP18)	0.022
<i>Casp3</i>	rs2696057	rs1859164 (SNP18)	0.014
<i>Casp3</i>	rs4647602	rs1859164 (SNP18)	0.017
<i>Casp3</i>	rs1405937	rs1859164 (SNP18)	0.029
<i>Casp3</i>	rs1049216	rs6968828 (SNP19)	0.026
<i>Casp3</i>	rs2696057	rs6968828 (SNP19)	0.041
<i>Casp3</i>	rs1049216	rs3807598 (SNP20)	0.010
<i>Casp3</i>	rs1405944	rs3807598 (SNP20)	0.025
<i>Casp3</i>	rs2696057	rs3807598 (SNP20)	0.026
<i>Casp9</i>	rs1052571	rs3757640 (SNP15)	0.024
<i>Casp9</i>	rs2308941	rs6968828 (SNP19)	0.042
<i>Casp10</i>	rs3900115	rs3779456 (SNP17)	0.008

^ap<0.05 shown and p<0.01 in bold

^bp-values uncorrected for multiple testing

Supplemental Table XIII. Results of GEE Analysis for gene-gene interactions of *HOX*, *IGFBP3* and mitochondrial mediated apoptotic variants in Hispanic population^{a,b}

Gene 1	SNP 1	Gene 2	SNP 2	p-value
<i>Bcl2</i>	rs2551402	HOXD	rs6749771 (SNP1)	0.045
<i>Bcl2</i>	rs1809319		rs1446575 (SNP2)	0.038
<i>Bcl2</i>	rs1809319		rs847146 (SNP8)	0.015
<i>Bcl2</i>	rs1809319		rs711812 (SNP10)	0.016
<i>Bcl2</i>	rs1809319		rs6758117 (SNP11)	0.012
<i>Bcl2</i>	rs1801018		rs6758117 (SNP11)	0.037
<i>Bid</i>	rs5747351		rs6749771 (SNP1)	0.015
<i>Bid</i>	rs3788284		rs6749771 (SNP1)	0.010
<i>Bid</i>	rs181410		rs1318778 (SNP3)	0.034
<i>Bid</i>	rs181399		rs1318778 (SNP3)	0.030
<i>Bid</i>	rs5747351		rs1867863 (SNP5)	0.027
<i>Bid</i>	rs181410		rs2113563 (SNP6)	0.045
<i>Bid</i>	rs3788284		rs2113563 (SNP6)	0.032
<i>Bid</i>	rs181399		rs2592394 (SNP7)	0.024
<i>Bid</i>	rs3788284		rs2592394 (SNP7)	0.018
<i>Casp3</i>	rs4647602		rs1318778 (SNP3)	<0.001
<i>Casp3</i>	rs4647602		rs2113563 (SNP6)	0.009
<i>Casp3</i>	rs2696057		rs2592394 (SNP7)	0.041
<i>Casp3</i>	rs4647602		rs2592394 (SNP7)	0.015
<i>Casp3</i>	rs2696057		rs741610 (SNP9)	0.008
<i>Casp3</i>	rs2696057	rs711812 (SNP10)	0.040	
<i>Casp9</i>	rs4233533	rs2428431 (SNP14)	0.043	
<i>Apaf1</i>	rs1866477	HOXA	rs3757640 (SNP15)	0.047
<i>Apaf1</i>	rs2278361		rs1859164 (SNP18)	0.043
<i>Apaf1</i>	rs7310804		rs6968828 (SNP19)	0.038
<i>Apaf1</i>	rs3782558		rs6968828 (SNP19)	0.010
<i>Apaf1</i>	rs1866477		rs6968828 (SNP19)	0.009
<i>Apaf1</i>	rs7968661		rs3807598 (SNP20)	0.023

^ap<0.05 shown and p<0.01 in bold

^bp-values uncorrected for multiple testing

