

Mutation screening of muscle development genes in patients with idiopathic clubfoot

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ABSTRACT

Background: Congenital idiopathic clubfoot is a very common musculoskeletal birth defect, but with no known etiology. Dietz *et al.* have shown possible linkage in chromosome 3 and 13 in a large, multigenerational family with congenital idiopathic clubfoot. Current evidence suggests that muscle development is impaired in patients with congenital idiopathic clubfoot, therefore we hypothesized that mutations in genes related to muscle development could be associated with this deformity. From the areas identified in the linkage study, candidate genes *SPRY2*, *RAF1*, *IQSEC1*, *LMO7*, and *UCHL3* were selected based upon their presence in skeletal muscle as well as their involvement in muscle development. **Methods:** The exons and splice sites of the five genes were screened via sequence-based analysis in a group of 24 patients with congenital idiopathic clubfoot. All single nucleotide polymorphisms (SNPs) found were compared to public databases to determine allelic frequency and amino acid modification. **Results:** While many SNPs were found, none proved to be significantly associated with the phenotype of congenital idiopathic clubfoot. The SNPs found were shown to be common amongst a non-clubfoot population and to follow the allelic frequency of the general population. **Conclusions:** Based upon these results, *SPRY2*, *RAF1*, *IQSEC1*, *LMO7*, and *UCHL3* are not likely to be the major causes of congenital idiopathic clubfoot. Given the complexity of myogenesis, many other candidate genes remain that could cause defects in the hypaxial musculature that is invariably observed in congenital idiopathic clubfoot. **Clinical Relevance:** This study further identifies genes which are unlikely to be the direct cause of congenital idiopathic clubfoot. It also helps to eliminate suspected genes found within the given bounds of chromosome 3 and 13.

Keywords: Clubfoot; Genetics; Muscle; *SPRY2*; *RAF1*; *IQSEC1*; *LMO7*; *UCHL3*

1. INTRODUCTION

Congenital idiopathic clubfoot is the most common pediatric musculoskeletal deformity affecting 1 - 7 per 1000 newborns. Despite extensive clinical and basic research, its etiology is unknown. However, a genetic component has been suggested. Twin studies have shown a concordance rate of 17% in monozygotic twins, 9% in dizygotic twins of the same sex, and 5% in dizygotic twins of opposite sex [1]. Also, first degree relatives of patient's with clubfoot have a higher probability of having clubfoot than the general population. For example, if a parent and one child are affected by clubfoot, then there is a 30% chance that subsequent children will also have clubfoot [2]. In addition, several studies have indicated the etiology of clubfoot may be caused by a single, major gene with incomplete dominant inheritance [3-5].

Based upon the growing evidence that a single gene may be responsible for the development of clubfoot, several studies have been reported looking at candidate genes. Genes involved in hind limb development (*PITX1*, *HOXD13*, *CAND2*, *WNT7A*), biotransformation of tobacco smoke (*NAT2*), apoptosis (*Casp8*, *Casp10*, and *CFLAR*), and collagen type IX (*COL9A1*) have all been evaluated and shown to not be related to the development of congenital idiopathic clubfoot [6-11]. Interestingly, Dietz *et al.* using a genome-wide screening of a four-generation family **Figure 1** with 13 affected and 41 unaffected members identified linkage regions on chromosome 3 and 13 (LOD score of 2.3) [12]. Therefore, the gene(s) causing idiopathic clubfoot could reside in these areas and a genetic mutation screening of candidate genes may provide insight into its etiology.

Several recent studies have reported evidence of a relationship between clubfoot and muscle development. Ippollito found muscle atrophy in patients with clubfoot and since this atrophy continues throughout life, impairment in muscle growth could be a likely pathological cause [13]. Ippollito *et al.* also described a "retracting fibrosis" that began early in fetal development; and beta-catenin levels have been shown to be increased in the connective

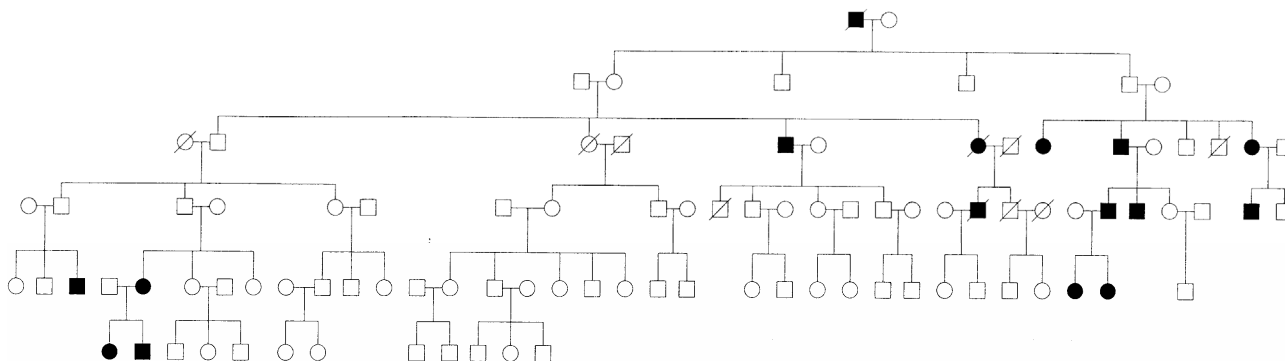


Figure 1. Omaha family pedigree.

tissue of clubfoot in a similar manner as in other fibro-proliferative diseases such as palmar fibromatosis [14, 15]. Furthermore, histological and electron microscopy studies have shown that muscle fibers exhibited a loss of organization and directionality, increased variability in the fiber size, increase in Type I fibers, and a decrease in Type II fibers [16-18]. The normal ratio of Type I:Type II fibers is 1:2, while in clubfoot patients the average ratio was 7:1, a 14-times decrease in Type II fiber [18]. Several other studies have found distinct neuromuscular abnormalities in muscle biopsies obtained from patients with congenital idiopathic clubfoot [16-23].

Based on these findings, 5 candidate genes—SPRY2, RAF1, IQSEC1, LMO7, and UCHL3—from the linkage areas by Dietz *et al.* were selected because their relationship with muscle development. Specifically, SPRY2 has been shown to be a negative regulator of fibroblast growth factor (FGF) signaling, involved in myogenesis, and leads to non-formation of myotubes if mutated [24,25]. RAF1 is involved in a pathway that regulates myoblast proliferation and differentiation. It also enhances the transcriptional activity of MyoD, a controller of muscle cell differentiation [26,27]. IQSEC1 is involved in the fusion of mammalian myoblasts, and when knocked down results in abnormal myotubes [28]. LMO7 interacts with beta-catenins and MyoD, and when deleted in mice, results in the development of dystrophic muscles [29]. Deletion of UCHL3 can cause a mild muscular dystrophy, and when deleted along with LMO7, the severity of muscle degeneration is increased [29,30]. This study was conducted based on the hypothesis that a primary mutation in any one of the five chosen candidate genes (SPRY2, RAF1, IQSEC1, LMO7, and UCHL3) could be associated with congenital idiopathic clubfoot.

2. MATERIALS AND METHODS

2.1. Study Subjects and Ascertainment Strategy

All protocols were approved by the human subjects review board of the University of Iowa Hospitals and Clinics. Informed consent was obtained from all participants.

Twenty-four patients with congenital idiopathic clubfoot without any other congenital abnormalities were identified. Patients with neuromuscular or other recognizable syndromes involving clubfoot were excluded. The Single Nucleotide Polymorphism reference through NCBI was used to identify any previously known SNPs and to compare the results of ethnically-matched allelic frequencies. All exons and splice-sites were screened.

2.2. DNA Extraction, PCR Amplification, and Direct Sequencing

DNA was extracted from cheek swabs using a standard DNA extraction method [31]. Saliva samples were used rather than venipuncture due to the pediatric population. The final concentration of the DNA was obtained using spectroscopy and all samples were diluted to 20 ng/ μ l and stored at 4°C during the duration of the experiments. The mRNA sequences for SPRY2, RAF1, IQSEC1, LMO7, and UCHL3 were obtained through a nucleotide search on the Ensembl website (<http://www.ensembl.org>), and the complete cds were used. At the time of sequencing, for SPRY2 (3 exons), the mRNA was 2126 bps long, the accession number is NM_005842. For RAF1 (18 exons), the mRNA was 3291 bps long, the accession number is NM_002880. For IQSEC1 (18 exons), the mRNA was 7229 bps long, the accession number is NM_001134382. For LMO7 (33 exons), the mRNA was 7253 bps long, the accession number is NM_005358. For UCHL3 (11 exons), the mRNA was 899 bps long, the accession number is NM_006002.

2.3. Sequence Analysis

After sequencing the forward strand first, the sequences were viewed using the program Sequencher and if a sequence variant was observed, then the reverse strand was sequenced to verify the result. Insertions, substitutions, deletions, and unknown nucleotides on one strand were checked against the other strand in a BLAST search. When a change was observed in the sequence of both strands, the change was labeled as a polymorphism. If it

was shown to be a common variant over a wide, ethnically matched population, and our allelic frequency was similar to the general population, the single nucleotide polymorphism (SNP) was considered unrelated to the development of clubfoot. However, if the SNP was not seen before, then the polymorphism would be considered novel and would then be run in a larger sample population and compared to an equally large control population to verify that the polymorphism was actually novel.

3. RESULTS

Based on sequence-comparison, no previously reported mutations in any of these genes were observed. Many missense and untranslated region variants were found, in addition to SNPs in the intronic areas of *SPRY2*, *RAF1*, *IQSEC1*, *LMO7*, and *UCHL3*. However, none of these variants differed compared to previously reported allelic frequencies or they were present in an untranslated region. In *SPRY2*, one missense Pro-Ser variant was found along with several SNPs in UTRs. In *RAF1*, one variant was found that was not previously reported, but it was in a UTR. In *IQSEC1*, only one missense Pro-Ser variant was found. In *LMO7*, three intronic and three missense variants were found. In *UCHL3*, three intronic splice site polymorphisms were found. With no new variant SNPs found, no separate control samples were run as the BLAST SNP program served as our control.

4. DISCUSSION

During the development of congenital idiopathic clubfoot, the musculoskeletal components of the lower extremity develop normally during the period of embryogenesis; however, between 12 and 32 weeks, as it has been observed by ultrasonography, the anatomy of the foot begin to deviate and leads to the deformity [32]. While congenital idiopathic clubfoot is usually described as a defect in the foot, the musculature between the knee and the ankle is affected to some degree, and most importantly, the size difference in the affected leg persists for the life of the patient [16,33]. Interestingly, relapses after correction significantly decreases after the child reaches 3 - 4 years of age [34]. This observations lead to the hypothesis that a genetic defect in muscle genes expressed early in life could be the cause of clubfoot.

The genes *SPRY2*, *RAF1*, *IQSEC1*, *LMO7*, and *UCHL3* selected for this study are involved in the embryonic development of muscle. They have been shown to regulate muscle formation through their respective affects on myotube formation, myoblast proliferation, and gene regulation. In addition, two of the genes (*LMO7* and *UCHL3*), when mutated, have been shown to result in a type of muscular dystrophy. This involvement in early muscle development and their location in the linkage

regions of chromosome 3 and 13 make them very appealing candidate genes for idiopathic clubfoot.

However, in this study, no mutations were observed that could explain the etiology of clubfoot. Of the many missense, UTR, and intronic variants found in the selected group of genes, none differed compared to the reported allelic frequencies. This showed that the genes *SPRY2*, *RAF1*, *IQSEC1*, *LMO7*, and *UCHL3* were unlikely to be directly involved with the development of congenital idiopathic clubfoot as the single major causative gene.

The limitations of this study were the relatively small sample size, intronic regions besides the splice-sites were not screened, and regulatory sequences for protein-DNA interactions were not investigated. Given that clubfeet of different syndromes appear and are treated similarly, it is possible that there are several distinct etiologies that combine to explain the development of congenital idiopathic clubfeet, in which case a large patient population would need to be screened in order to achieve an adequate representation of all possible pathology. However, based on our hypothesis that a single, major gene mutation is causing congenital idiopathic clubfoot, screening small cohorts is acceptable because the predicted mutation should be in the vast majority of patients screened. Also, our study is limited by only screening candidate genes selected from a linkage region specified by Dietz, *et al.* This region did not have a LOD score that exceeded 3, which is the standard score for linkage significance. However, we believed the linkage still deserved to be further investigated as it was the first and only genome-wide linkage analysis conducted in clubfoot families, and the LOD score > 2 still encouraged investigation. Lastly, we also only screened muscle development genes. While other proposed causes, such as neurologic development, might also contribute to clubfoot development, these were not screened based on the hypothesis that clubfoot may be primarily a muscle disorder.

In summary, screening of the exons and splice sites of *SPRY2*, *RAF1*, *IQSEC1*, *LMO7*, and *UCHL3* demonstrated no mutations associated with the phenotype of congenital idiopathic clubfoot. This result does not exclude the possibility that there exist genetic variants in the studied genes that affect clubfoot in a more complicated manner through protein-protein interactions or enhancer and suppressor sites. Defects in regulatory pathways or proteins that interact with these genes could act on them and lead to clubfoot. Thus, these muscle development genes would be an important, but not direct cause of clubfoot. Given the complexity of myogenesis, many regulatory candidate genes remain that could cause developmental defects in the hypaxial musculature that is invariably observed in clubfoot. Lastly, future research will focus on performing genome-wide analysis of the Omaha pedigree **Figure 1** again with new technology

that was not available to Dietz, *et al.* The family shows a strong genetic component that will increase the possibility of finding a potentially causative gene for the development of clubfoot.

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