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*J Pediatr Orthop*. Author manuscript; available in PMC 2010 October 01.

Published in final edited form as:
*J Pediatr Orthop*. 2010 ; 30(3): . doi:10.1097/BPO.0b013e3181d35e3f.

# Evaluation of Embryonic and Perinatal Myosin Gene Mutations and the Etiology of Congenital Idiopathic Clubfoot

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## Abstract

**Background—**Congenital idiopathic clubfoot is the most common musculoskeletal birth defect developing during the fetal period, but with no known etiology. MYH 2, 3, 7, and 8 are expressed embryonically or perinatally, the period during which congenital idiopathic clubfoot develops; are all components of Type II muscle, which is consistently decreased in clubfoot patients; and are associated with several muscle contracture syndromes that have associated clubfoot deformities. In this study, we hypothesized that a mutation in an embryonic or perinatal myosin gene could be associated with congenital idiopathic clubfoot.

**Methods—**We screened the exons, splice sites, and predicted promoters of 24 bilateral congenital idiopathic clubfoot patients and 24 matched controls in MYH 1, 2, 3, and 8 via sequence-based analysis, and screened an additional 76 patients in each discovered SNP.

**Results—**While many SNPs were found, none proved to be significantly associated with the phenotype of congenital idiopathic clubfoot. Also, no known mutations that cause distal arthrogryposis syndromes were found in the congenital idiopathic clubfoot patients.

**Conclusion—**These findings demonstrate that congenital idiopathic clubfoot has a different pathophysiology than the clubfoot seen in distal arthrogryposis syndromes, and defects in myosin are most likely not directly responsible for the development of congenital clubfoot. Given the complexity of early myogenesis, many regulatory candidate genes remain that could cause defects in the hypaxial musculature that is invariably observed in congenital idiopathic clubfoot.

**Clinical Relevance—**This study further differentiates congenital idiopathic clubfoot as distinct from other complex genetic syndromes that can present with similar deformities, and thus facilitates further research to improve the clinical diagnosis and treatment of congenital idiopathic clubfoot.

### Keywords

arthrogryposis; clubfoot; genetics; myosin

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## Introduction

Congenital idiopathic clubfoot is the most common musculoskeletal birth defect affecting 0.3 to 7.8 per 1000 new born.[1–3] Most studies have found males to be at least twice as likely to be affected as females.[4] A genetic component to the etiology of clubfoot has been suggested. In twin studies, dizygotic twins only had a concordance rate of 2.9%, while monozygotic twins had a significantly higher 32.5% concordance rate.[5] Also, first degree relatives of clubfoot patients have a significantly higher probability of also having clubfoot when compared to the general population. For example, if both parents or one child and another family member both had clubfoot, then the chance that the parents would have a child with clubfoot is 10–20%.[6] In addition, several studies have indicated the etiology of clubfoot may be caused by a single, major gene with incomplete dominant inheritance.[6,7]

Histological and electron microscopy studies have provided valuable insight into the pathological changes in patients with congenital idiopathic clubfoot, in which decreased muscle size in the effected leg is a consistent characteristic. The primary findings of these studies demonstrated that the muscles 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.[8–10] 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 fibers.[10] Handelsman hypothesized that the increase slow-twich, high tension fibers may be overactive and create a persistent deforming force leading to clubfoot.[10] Several other studies have found genetic and distinct neuromuscular abnormalities in muscle biopsies obtained from patients with congenital idiopathic clubfoot.[8–15]

Myosin is the primary contractile protein in muscle, and consists of different isoforms that are selectively expressed or suppressed during specific stages in development. Based on ultrasound, clubfoot arises early in fetal development during weeks 16–30. While our current knowledge of the functions of MYH 1, 2, 3, and 8 is very limited, we do know that they are the specific myosin isoforms that are expressed during this particular window. Interestingly, several recent studies have reported evidence of a relationship between embryonic or perinatal myosin and clubfoot. Distal arthrogryposis is a congenital contracture syndrome that often presents with camptodactyly and clubfeet. Toydemir et al. found that multiple mutations in embryonic MYH3 cause Freeman-Sheldon syndrome (FSS) and one-third of Sheldon-Hall syndrome (SHS) cases, which both often present with clubfeet.[15] In addition, Veugelers et al. found that a mutation in perinatal MYH8 causes trismus-pseudocamptodactyly syndrome, which can also present with clubfeet.[14] Martinsson et al. found mutations in MYH2 cause hereditary inclusion body myopathy, which presents as progressive muscle weakness.[13] Given that most clubfeet develop during the 2nd and third trimester of pregnancy, particular relevance is given to myosin genes that are expressed during this time, such as MYH 1, 2, 3, and 8.

This study was conducted based on the hypothesis that a primary mutation in an embryonic or perinatal myosin gene could be associated with congenital idiopathic clubfoot. In addition, we also hypothesize that congenital idiopathic clubfoot might share a mutation with other muscle contracture syndromes that present with clubfoot, such as Freeman-Sheldon Syndrome. In screening MYH 1, 2, 3, and 8, we hope to find the single, major gene that through incomplete dominant inheritance explains the development of congenital idiopathic clubfoot.

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## Materials and Methods

### 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. One hundred patients with bilateral congenital idiopathic clubfoot without any other congenital abnormalities were identified. Patients with neuromuscular or other recognizable syndromes involving clubfoot were excluded. One hundred gender and ethnically-matched individuals without any foot deformities were used as controls. All exons, splice-sites, and promoters were screened using 24 clubfoot patients and controls, and the remaining patients and controls were used when SNPs were found to provide an expanded sample size. For rs719277 and rs3744565, an additional 100 patients and controls were screened (total of 200 patients and 200 controls) because a very weak significant relationship was seen in the first 100 patients. The rationale for screening these relatively small sample sizes is that the current literature favors a single, major gene that causes clubfoot; therefore, we are looking for a mutation that will be present in the vast majority of patients with clubfoot. If our hypothesis had been that clubfoot is polygenic or multifactorial, then our sample sizes would have been significantly larger.

### DNA Extraction, PCR Amplification, and Direct Sequencing

Seven to 14 mL of whole blood was collected from each participant by venipuncture. DNA was extracted using a previously described protocol.[16] The concentration of each sample was 20 ng/μL, and the samples were stored at 4°C. The mRNA sequences for MYH 1, 2, 3, and 8 were obtained through a nucleotide search on the NCBI website (http://www.nbci.nih.gov), and the complete cds were used. For MYH1 (40 exons), the mRNA was 6,024 bps long, the accession number was NM_005963. For MYH2 (40 exons), the mRNA was 6,084 bps long, the accession number was NM_017534. For MYH3 (40 exons), the mRNA was 6,016 bps long, the accession number was NM_002470. For MYH8 (39 exons), the mRNA was 6,019 bps long, the accession number was NM_002472.

Predicted promoter regions were obtained for MYH 1, 2, 3, and 8 using http://www.cbs.dtu.dk/services/Promoter. Primers were designed for the promoters and each exon of MYH 1, 2, 3, and 8 using the Primer3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The primers were compared to the "hstg" database using BLAST search to ensure the primers annealed only where they were designed to anneal. After the primers were obtained, they were diluted to 20 μM and stored at −20°C.

The general protocol used for the PCR reactions per sample was 5 μL 10X Qiagen PCR Buffer, 1 μL dNTP (10mM each), 3 μL forward primer (20 μM), 3 μL reverse primer (20 μM), .5 μL *Taq* DNA polymerase (5 U/μL), 10 μL Q solution, 25 μL $dH_2O$, and 2.5 μL template DNA. The general cycling conditions used for the PCR reactions was activation of the *taq* polymerase at 95°C for 5 minutes and initial denaturation at 95°C for 2 minutes, 30 cycles were performed at 94°C for 1 minute, 57°C for 1 minute, and 72°C for 1 minute, followed by a last extension at 72°C for 10 minutes. The PCR products were purified using the QIAquick PCR Purification Kit (Cat. No. 28104) following the protocol enclosed in the kit. 20 μL of the purified DNA products were sent to the University of Iowa DNA facility for sequencing.

### Sequence Analysis

After sequencing, the two sequences for each primer set were analyzed using both BLAT and BLAST searches. Both the positive and negative strands of each sample were simultaneously inputted into a BLAT search, and the percent homology and the position of

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the PCR amplification were recorded. 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. Finally, we searched the sequences of the control subjects at the locations polymorphisms were found and observed whether there were similar changes in the sequences of the control patients. Chi-square analyses were performed to compare the genotype frequencies of the SNPs found in patients and controls.

## Results

Based on sequence-comparison, many silent and missense variants were found, in addition to SNPs found in the intronic areas of MYH 1, 2, 3, and 8 (Table 1). In MYH1, only silent sequence variants were found. In MYH2, three intronic splice site polymorphisms were found, but they did not associate significantly with the phenotype of clubfoot when compared to controls. In MYH3, four intronic splice site polymorphisms and one exonic polymorphism with an A-to-T amino acid change were found, but none differed significantly compared to controls. In MYH8, one splice site polymorphism, one exonic polymorphism with an R-to-W amino acid change, and one polymorphism in the predicted promoter were found, but again none differed significantly compared to controls. None of the MYH3 and MYH8 SNPs Toydemir and Veugeler found to cause Freeman-Sheldon and trismus-pseudocamptodactyly syndrome were found in our patients.

## Discussion

During the development of congenital idiopathic clubfoot, the musculoskeletal components of the lower extremity develop normally prior to 16 weeks; however, between 16 and 30 weeks, as it has been observed by ultrasound, the anatomy of the foot begin to deviate and leads to the deformity. 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.[8,17] Interestingly, studies have found that recurrence of clubfoot after correction significantly decreases after the child reaches 3–4 years of age.[18] This observation lead to the hypothesis that a genetic defect in muscle genes expressed early in life could be the cause of clubfoot. When normal adult myosin gene expression takes over, the viscoelastic properties of the muscle change and the deforming factor is no longer present. In addition, since the clubfeet observed in distal arthrogryposis is essentially indistinguishable from congenital idiopathic clubfeet, they could share the same pathogenic mechanisms.

During normal muscle human development, expression of certain myosin genes (MYH 1, 2, 3, 8) is restricted to the embryonic period of development prior to birth, and quickly declines after birth as adult myosin genes are upregulated.[19] The individual functions of MYH 1, 2, 3, and 8 have not been described beyond the fact that these are the only four myosin isoforms that are specifically expressed during the embryonic period. Myosin is the primary contractile protein in muscle that transforms chemical to mechanical energy, and is a hexameric protein formed by two heavy chains and two light chains. During fetal weeks 16–30, the only myosin isoforms that are expressed are MYH 1, 2, 3, and 8, while all other known isoforms are actively suppressed. Given that clubfoot develops during a specific window in fetal development, only those myosin isoforms that are selectively expressed during this period are relevant candidate genes.

MYH3 mutations that cause FSS create structural changes that disrupt the nucleotide binding site or the catalytic groove; therefore, these relatively small changes impair the contractile ability of the sarcomeres.[15] In an animal model using *Drosophila*, adult myosin

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genes were predicted to be up-regulated when embryonic myosin genes are dysfunctional.[20,21] Despite the attempted compensation, muscle function is impaired due to the different basal ATPase abilities of the embryonic vs. adult myosins. As seen in FSS and SHS, even small defects in embryonic myosin proteins cause severely deforming forces, even though the myosin genes are still expressed and produced.

Similar to FSS and SHS, myosin abnormalities could introduce a deforming force during an essential embryonic or fetal development in congenital idiopathic clubfoot. Since no mutations in MYH 2, 3, and 8 were found in congenital idiopathic clubfoot patients, we conclude that while congenital congenital idiopathic clubfoot shares a common deformity with clubfeet found in some contracture syndromes, congenital congenital idiopathic clubfoot most likely possesses a distinct etiopathogensis.

The limitations of this study were the relatively small sample sizes, only bilateral cases were included, intronic regions besides the promoters and splice-sites were not screened, and regulatory 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 each possible pathology. Also, our study is limited by only screening myosin genes, while neurologic development might also contribute to clubfoot development. Neuromuscular junction defects lead to histological similarities with the histopathology of clubfoot, but based on the known connection between distal arthrogryposis syndromes and myosin genes, and our hypothesis that clubfoot is primarily a muscular disorder, we did not screen genes related to neuromuscular development. 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. If we believed the etiology of clubfoot to be polygenic, and that mutations in several genes could all lead to the development of clubfoot, then significantly larger cohorts would need to be screened in order to discern significant differences in the genotype frequencies amongst the numerous polymorphisms. Also, the rationale for only including bilateral cases is that we wanted patients who had the same phenotype and biologically, bilateral cases best fit the hypothesis of a single gene mutation. All known myosin mutations have led to bilateral phenotypic changes, which led us to limit our patients accordingly. We planned to investigate in subsequent experiments whether unilateral clubfoot cases were due to incomplete gene expression within the body, but this would be a much more complicated task and beyond the goals of the current study.

In summary, screening of the exons, splice sites, and predicted promoters of MYH 1, 2, 3, and 8, 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 more complicated manner through protein-protein interactions or enhancer and suppressor sites. Defects in a myosin regulatory pathway or protein that interacts with myosin could act on normal myosin leading to clubfoot and therefore myosin would be an important but not the direct cause of clubfoot. Also, patients with congenital idiopathic clubfoot did not share any MYH mutations with the other known distal arthrogryposis syndromes. These findings demonstrate that congenital idiopathic clubfoot has a different pathophysiology than the clubfoot seen in distal arthrogryposis syndromes. 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.

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## Acknowledgments

Financial support was provided by the Howard Hughes Medical Institute.

The authors would like to thank the Carver College of Medicine Research Program for their financial support. Dr. Val Sheffield is an investigator of the Howard Hughes Medical Institute.

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**Table 1**

SNP locations, genotype frequencies, amino acid changes, and ID numbers for mutations in MYH 2, 3, and 8.

| Gene | Exon/Intron | Patient Genotype Frequencies (% homozygous wild type, heterozygote, homozygous variant) | | | Control Genotype Frequencies (% homozygous wild type, heterozygote, homozygous variant) | | | Amino Acid Change | ID # |
|---|---|---|---|---|---|---|---|---|---|
| **MYH2** | Intron | 28 | 54 | 18 | 33 | 42 | 24 | | rs719277 |
| **MYH2** | Intron | 24 | 48 | 28 | 33 | 46 | 21 | | rs3744565 |
| **MYH2** | Intron | 42 | 40 | 19 | 32 | 38 | 30 | | rs2277651 |
| **MYH3** | Intron | 27 | 57 | 16 | 31 | 60 | 9 | | rs2239934 |
| **MYH3** | Intron | 50 | 39 | 10 | 45 | 41 | 14 | | rs2285468 |
| **MYH3** | Intron | 31 | 59 | 9 | 32 | 53 | 14 | | rs876660 |
| **MYH3** | Exon | 40 | 53 | 7 | 37 | 47 | 17 | A-to-T | rs2285477 |
| **MYH3** | Intron | 0 | 100 | 0 | 0 | 100 | 0 | | rs2239936 |
| **MYH8** | Intron | 52 | 20 | 28 | 44 | 23 | 32 | | rs4791407 |
| **MYH8** | Exon | 25 | 75 | 0 | 28 | 72 | 1 | R-to-W | rs8069834 |
| **MYH8** | Intron | 16 | 75 | 9 | 17 | 81 | 2 | | rs2277648 |