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SYMPOSIUM: CLUBFOOT: ETIOLOGY AND TREATMENT

Skeletal Muscle Contractile Gene (TNNT3, MYH3, TPM2) Mutations Not Found in Vertical Talus or Clubfoot

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Abstract Arthrogryposis presents with lower limb contractures that resemble clubfoot and/or vertical talus. Recently, mutations in skeletal muscle contractile genes MYH3 (myosin heavy chain 3), TNNT3 (troponin T3), and TPM2 (tropomyosin 2) were identified in patients with distal arthrogryposis DA2A (Freeman-Sheldon syndrome) or DA2B (Sheldon-Hall syndrome). We asked whether the contractile genes responsible for distal arthrogryposis are also responsible for cases of familial clubfoot or vertical talus. We determined the frequency of MYH3, TNNT3, and TPM2 mutations in patients with idiopathic clubfoot, vertical talus, and distal arthrogryposis type 1 (DA1). We resequenced the coding exons of the MYH3, TNNT3, and TPM2 genes in 31 patients (five with familial vertical talus, 20 with familial clubfoot, and six with DA1). Variants were evaluated for segregation with disease in additional family members, and the frequency of identified variants was determined in a control population. In one individual with DA1, we identified a de novo TNNT3 mutation (R63H) previously identified in an individual with DA2B. No other causative mutations were identified, though we found several previously undescribed single-nucleotide polymorphisms of unknown importance. Although mutations in MYH3, TNNT3, and TPM2 are frequently associated with distal arthrogryposis syndromes, they were not present in patients with familial vertical talus or clubfoot. The TNNT3 R63H recurrent mutation identified in two unrelated individuals may be associated with either DA1 or DA2B.

Level of Evidence: Level II, prospective study. See the Guidelines for Authors for a complete description of levels of evidence.

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Each author certifies that his or her institution has approved the human protocol for this investigation, that all investigations were conducted in conformity with ethical principles of research, and that informed consent for participation in the study was obtained.

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Introduction

Idiopathic clubfoot is one of the most common congenital musculoskeletal anomalies, with a worldwide incidence of 1 in 1000 births. Clubfoot is most frequently an isolated condition and the cause is currently unknown [28]. Approximately 80% of clubfeet occur as an isolated birth defect and are considered idiopathic [27], while the remainder have a known syndrome, musculoskeletal disorder, or chromosomal etiology. Genetic factors play a role in the etiology of idiopathic clubfoot, as nearly 25% of all cases are familial [17]. Despite the relatively high frequency of idiopathic clubfoot and the strong evidence for a genetic basis of the disorder, no causative genes have yet been identified.

Clubfoot may occur with additional malformations, chromosomal abnormalities, or known genetic syndromes, including distal arthrogryposis, a condition for which mutations in several skeletal muscle contractile genes have recently been identified.

Clubfoot or vertical talus occurs in nearly all patients with distal arthrogryposis and is often phenotypically similar to idiopathic clubfoot. As a class, distal arthrogryposis may result from mutations in five skeletal muscle contractile or regulatory genes: myosin heavy chain (MYH3, MYH8), troponin I (TNNI2), troponin T (TNNT3), and tropomyosin (TPM2) [22, 25, 26]. Mutations in these genes have been described most frequently in patients with the more severe forms of distal arthrogryposis, including Freeman-Sheldon Syndrome (DA2A) and Sheldon-Hall Syndrome (DA2B) [21], in which facial contractures are also present. To our knowledge, only a single mutation in TPM2 [22] has been described in patients with the mildest form of distal arthrogryposis, distal arthrogryposis type 1 (DA1), in which contractures are limited to the hands and feet. Patients with DA1 are also less likely to have scoliosis, strabismus, and vertical talus, though the classification of these disorders currently relies on clinical criteria that are often difficult to apply in practice [2, 23].

One of the genes responsible for distal arthrogryposis, MYH3, is expressed during the embryonic period [11], while other myosin-heavy chain genes (MYH1, MYH2, and MYH4) become predominant during later development and adulthood. This temporal expression pattern supports a theory that congenital contractures may result from a period of fetal akinesia [14]. However, recent in vitro evidence suggests human mutations in TNNT3, TNNI2, and TPM2 increase the contractility of fast-twitch muscle fibers [19], with the result being that the contracture forms because of excess contractility. Muscle abnormalities limited to the fetal period are consistent with the natural history of most cases of successfully treated idiopathic clubfoot, in which the contracture does not recur and the foot demonstrates minimal weakness [6].

Because of the phenotypic similarities between idiopathic clubfoot and congenital vertical talus and the foot phenotypes described in individuals with distal arthrogryposis [3], the contractile genes responsible for distal arthrogryposis are plausible candidate genes for these more common distal limb birth defects. We therefore sought to (1) determine the frequency of MYH3, TPM2, and TNNT3 gene mutations in patients with familial idiopathic clubfoot and vertical talus, (2) determine the frequency of mutations in these same genes in patients with the mildest form of distal arthrogryposis, DA1, and (3) confirm the frequency of any gene abnormalities found in a group of ethnically matched control patients without any foot anomalies.

Materials and Methods

We identified 31 patients with phenotypically related feet: 20 with familial idiopathic clubfoot, five with familial vertical talus and/or clubfoot [13], and six with distal arthrogryposis type 1A (DA1) (Table 1). Only one DA1 patient had a positive family history; the other five were sporadic cases. The patients with idiopathic clubfoot consisted of individuals with more than three affected family members (Table 1). Most cases were bilateral, but there

Table 1. Description of probands and families studied

Phenotype	# Families		# of affected individuals per family (description)
Vertical talus	5		2–4
		Fam 5865	2 (one with vertical talus; one with left vertical talus and right metatarsus adductus and bilateral ulnar deviated hands)
		Fam 2222	3
		Fam 1294	5 (one with left clubfoot and right vertical talus)
		Fam 1111	4 (two with bilateral clubfoot)
		Fam 6–999	2
Clubfoot	20		3–10
Distal arthrogryposis type 1 (DA1)	6		1–10

were several familial cases of clubfoot with both unilateral and bilateral subjects. All clubfeet in this study were severe and classified as grade 4 using the classification system of Dimeglio et al. [7]. A detailed description of the vertical talus families has been previously described [8]. All clubfeet were treated successfully using the Ponseti method [16] and all of the vertical talus feet were corrected using a new method of treatment described by Dobbs et al. [9, 10]. The study protocol was approved by the Washington University Human Research Protection Office, and all subjects and/or their parents gave informed consent.

DNA was obtained from either peripheral blood or from a spit sample (OraGene™ Self-Collection kit; Genotek, Ottawa, Canada) of probands and their affected and unaffected relatives when available. Subjects were excluded from the study if they had additional nonskeletal anomalies or known genetic syndrome. Affected individuals were examined by a single orthopaedic surgeon (MBD), and most were also evaluated by a clinical geneticist. Control samples consisted of individuals with epilepsy.

The exons containing coding regions as well as 5′ and 3′ untranslated regions of MYH3, TNNT3, and TPM2 genes were sequenced with the BigDye™ Terminator Cycle Sequencing Ready Reaction sequencing kit (Applied Biosystems, Foster City, CA). Each exon was sequenced in both forward and reverse directions for each individual. Reactions were fractionated on an ABI PRISM® 377 DNA sequencer (Applied Biosystems) and analyzed with Sequencher™ 4.2 (Gene Codes Corp, Ann Arbor, MI). Sequence was analyzed with Sequencher™ 4.1 software (Gene Codes Corp). Prediction of functional effects of polymorphisms/variants was performed using PolyPhen (http://genetics.bwh.harvard.edu/pph/index.html) [24].

Results

Analysis of the MYH3 gene in the familial clubfoot and vertical talus patients demonstrated several rare single-nucleotide variants that were likely non-disease-causing due to the lack of segregation with disease in the families studied (Table 2). One rare missense mutation, resulting in the E1149Q amino acid substitution, is of unknown importance, as it occurred only in one clubfoot patient; other affected family members declined to participate in the study. Sequencing of the TNNT3 and TPM2 genes did not reveal any mutations in either the familial clubfoot or vertical talus patients.

Sequencing of TNNT3 revealed a single mutation causing an R63H amino acid substitution in a child with isolated hand and foot contractures (Pt 5186001) (Fig. 1A). The patient had bilateral severe equinovarus contractures (Fig. 1B). No facial contractures or scoliosis were present; thus the patient was considered to have distal arthrogryposis type I (Fig. 1C). The mutation was not present in the DNA of either unaffected parent (Fig. 2) or in any of the other five probands with DA1. In addition, sequence analysis of the TPM2 and MYH3 genes revealed no mutations in the DA1 patient cohort.

None of the rare MYH3 variants found in the familial clubfoot and vertical talus patients were identified in a group of 96 ethnically matched control samples without foot anomalies.

Discussion

Because of the phenotypic similarities between idiopathic clubfoot and congenital vertical talus and the foot phenotypes described in individuals with distal arthrogryposis [3], the contractile genes responsible for distal arthrogryposis appeared plausible candidate genes for these more common distal limb birth defects. We therefore asked whether the contractile genes responsible for distal arthrogryposis are also responsible for cases of familial clubfoot or vertical talus.

The relatively small number of familial cases available for analysis is a limitation of the study. In the 25 patients with familial idiopathic clubfoot or vertical talus, we found no mutations in the genes' contractile proteins. Although we failed to find rare disease-causing mutations in these genes, we have not excluded the possibility that clubfoot is due to the inheritance of common polymorphisms that increase susceptibility to these conditions. These studies

Table 2. Rare, probable benign variants in MYH3 identified in this study

Nucleotide	Amino acid	Frequency in controls	Prediction of effect (PolyPhen)	Notes
1517A>G	N483S	0/96	Benign	In patient with DA2B, also in unaffected mother
3486C>T	R1137H	0/96	Probably damaging	rs12941197, does not segregate with disease
3514G>C	E1149Q	0/176	Benign	In patient with clubfoot, other affected family members declined to participate
3661G>A	A1198T	0/96	Benign	In patient with clubfoot, does not segregate with disease
4151C>T	A13261 V	0/96	Benign	In patient with clubfoot, does not segregate with disease

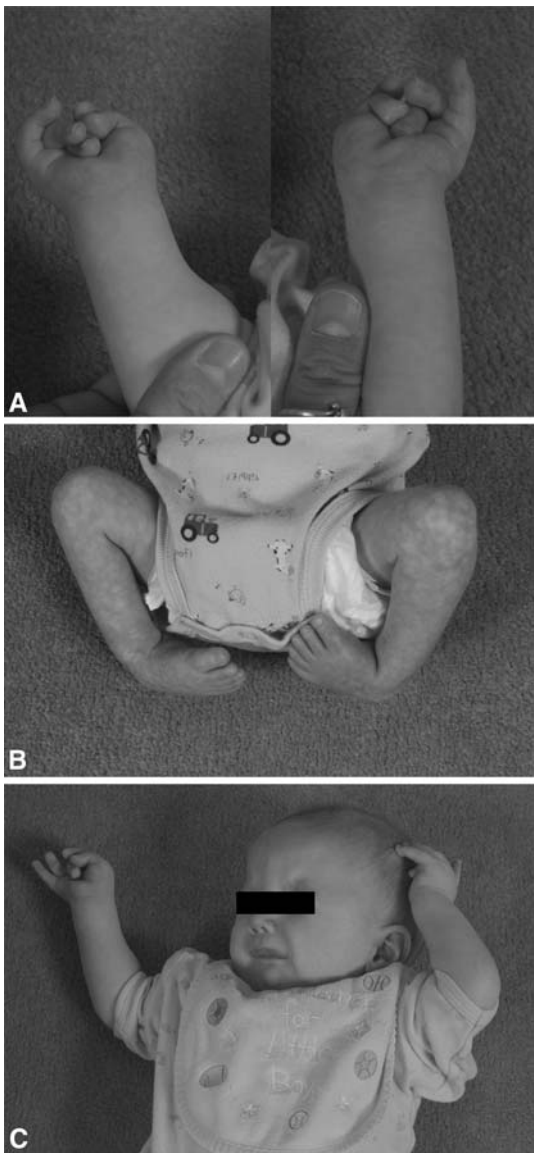

Fig. 1A–C Photographs show male proband at 3 months of age with the diagnosis of DA1. (**A**) The right and left hands demonstrate clenched fists and adducted thumbs. (**B**) Bilateral severe clubfoot deformities are evident with fixed forefoot adduction, hindfoot varus, and hindfoot equinus contractures. (**C**) There is no evidence of abnormal facies.

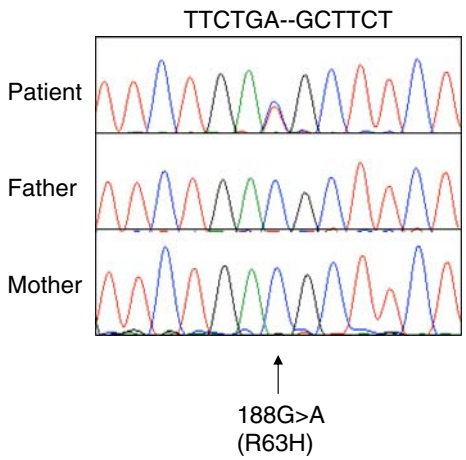

Fig. 2 A chromatogram shows the presence of the missense mutation in TNNT3, which results in the R63H amino acid substitution in a patient with distal arthrogryposis (DA1). The mutation was de novo, as it was not present in either parent. A = adenine (green); T = thymine (red); G = guanine (black); C = cytosine (blue).

would require a genetic association study with a much larger patient population.

Compared to the distal arthrogryposis syndromes that involve muscles in both upper and lower extremities as well as other parts of the body, isolated idiopathic vertical talus and clubfoot affect only the lower extremities. Lack of mutations in these skeletal muscle contractile genes in clubfoot and vertical talus may therefore not be unexpected since there does not appear to be selective expression of these contractile genes in the lower extremity. Several studies have identified genes whose expression is higher in the lower extremity [18, 20], yet many of these genes are transcription factors, and none are genes encoding contractile elements.

Due to the large population of clubfoot patients evaluated at our center, we have begun to recognize and diagnose individuals with mild forms of distal arthrogryposis. We identified a de novo TNNT3 R63H mutation in an infant with bilateral clubfoot and hand contractures but with no apparent facial weakness. This mutation was previously described in three individuals of a family with DA2B [23]. That the recurrent TNNT3 R63H mutation may cause either DA1 or DA2B suggests there may be other factors modifying the phenotype. Phenotypic variability in distal arthrogryposis occurring with identical MYH3 mutations has been described previously [25]. However, it is admittedly difficult to diagnose mild forms of facial weakness, particularly in infancy, and thus it may be difficult to clearly distinguish patients with DA1 (affecting only the hands and feet) from other types of distal arthrogryposis.

Less is known about the genetic cause of distal arthrogryposis type 1 (DA1) compared to the other more severe forms of arthrogryposis. The prevalence of DA1 is likely higher than the other forms of arthrogryposis [15], occurring in 1:10,000 to 1:50,000 births [1]. While mutations in MYH3 account for 93% of patients with Freeman-Sheldon syndrome (DA2A) and 32% of patients with Sheldon-Hall syndrome (DA2B) [25], the frequency of MYH3 mutations in patients with DA1 appears to be low; we found no mutations in this gene in any of our six patients. Furthermore, after screening for mutations in MYH3, TNNT3, and TPM2, a mutation was found in only one of six patients. Thus, it is likely that additional genes, which remain to be identified, are responsible for these milder conditions.

As has been previously noted, the myosin heavy chain genes are large, multiexonic genes that appear to harbor a relatively high rate of single-nucleotide polymorphisms and are susceptible to spontaneous and recurrent disease-causing mutations [5, 25]. We identified several variants of unknown significance during the study, either because these mutations did not segregate with disease or because parental samples were unavailable. The effects of these nonsynonymous amino acid substitutions were predicted to be benign on the basis of prediction of functional effects by PolyPhen [24]. One known polymorphism (rs12941197) that causes an R1137H amino acid substitution was predicted by PolyPhen [24] to be probably damaging but did not segregate with clubfoot as it was present in an unaffected parent and absent in an affected sister. This single-nucleotide polymorphism was not identified in 96 ethnically matched control samples and the frequency of rs12941197 polymorphism is not listed in the dbSNP Web site (http://www.ncbi.nlm.nih.gov/SNP). The significance of the E1149Q nucleotide substitution identified in one clubfoot patient is unknown. While the glutamate at position 1149 is conserved across all species and is conserved in other MYH family members (MYH1 and MYH2), substitution of a glutamine at this position is predicted by PolyPhen [24] to have benign structural and functional consequences. It is possible some of these variants may not be specifically disease-causing yet may still contribute to the phenotype as disease modifiers. Additional studies on larger numbers of patients will be required to understand the potential minor effects of some of these variants.

Although the genetic basis of idiopathic clubfoot is currently unknown, it is likely this condition represents a heterogeneous group of patients. This statement is based on our recent description of a variety of known disorders, including arthrogryposis, causing clubfoot in a series of 357 patients [12]. Clubfoot appears to represent a common phenotype for disruption anywhere along the neuromuscular unit, including the brain, spinal cord, nerve, or muscle. Irrespective of the genetic etiology, it is clear the foot contractures (clubfoot or vertical talus) in patients with distal arthrogryposis syndromes can frequently be treated successfully with the Ponseti technique (or modifications thereof) that was originally developed for idiopathic clubfoot [4].

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