Evaluation of CAND2 and WNT7a as Candidate Genes for Congenital Idiopathic Clubfoot

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Abstract Congenital idiopathic clubfoot is a common pediatric musculoskeletal deformity with no known etiology. The deformity reportedly follows a Mendelian pattern of inheritance. Recent work has demonstrated linkage in chromosome 3 and 13 in a large, multigeneration, highly penetrant family with idiopathic clubfoot. From the linkage region on chromosome 3, we selected the candidate genes CAND2 and WNT7a, which are involved in lower extremity development, and hypothesized mutations in these genes would be associated with the phenotype of congenital idiopathic clubfoot. The CAND2 gene was sequenced in 256 clubfoot patients, and 75 control patients, while WNT7a was screened using 56 clubfoot patients and 50 control patients. We found a polymorphism in each gene, but the single nucleotide change in CAND2 was a silent

mutation that did not alter the amino acid product, and the single nucleotide change in WNT7a was in the upstream, non-coding or promoter region before the start codon. Based on these results it is unlikely CAND2 and WNT7a are the major genes that causes clubfoot, however WNT7a might be one of many genes that could increase susceptibility to develop clubfoot but do not directly cause it.

Introduction

Congenital idiopathic clubfoot is a relatively common deformity found in one to two per 1000 Caucasian newborns and in six to eight per 1000 in Hawaiian/Maori individuals [2–4]. One study suggests males are at least twice as likely to be affected as females [11]. A genetic component to the etiology of clubfoot has been suggested by several studies. In twin studies, dizygotic twins had a concordance rate of 2.9%, while monozygotic twins had a 32.5% concordance rate [15]. Also, first-degree relatives of clubfoot patients have a higher probability of 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% to 20% [14]. In addition, several studies suggest the etiology of clubfoot may be caused by a single major gene [9, 14]. Rebbeck et al. used a regressive logistic model of complex segregation analysis and concluded a single gene expressing itself via incomplete dominant inheritance best fit the data [9]. The Maori and Polynesian populations demonstrate a similar single gene inheritance model under complex segregation analysis according to Chapman et al. [3]. Yang et al. also identified the same single gene inheritance model for Hawaiian and Caucasian groups [16].

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Each author certifies that his or her institution has approved the animal and human protocol for this investigation and 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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Numerous genes with diverse biological roles are currently being analyzed for association with clubfoot. Dobbs et al. reported mutations in *HOXD10*, which is a transcription factor with a role in limb development, were associated with clubfoot in three families [6]. Hecht et al. found mutations in *NAT2*, which plays a role in biotransformation of tobacco smoke, may be associated with clubfoot [7]. These studies support the argument that continued research is needed to illuminate the genetic foundation of clubfoot. While those genes have been associated with clubfoot in specific families, no genes have been associated using large populations of unrelated clubfoot patients.

Using a genome-wide screen of a four-generation family with 13 affected and 41 unaffected members, Dietz et al. [5] reported two regions on chromosomes 3 and 13 with LOD (logarithm [base 10] of odds) scores greater than two. They suggested clubfoot related to a single major gene, with multi-gene susceptibility. The general criteria for significance in LOD scores is if they exceed 3 (odds of 1 in 1000 for a false positive result of the gene located in this region); however, given the high quality of the pedigree, the complexity of the inheritance for clubfoot, and this being the only published genome-wide linkage study, we believe it is reasonable to pursue this finding even with a relatively low LOD score of 2.3 (odds of 1 in 200 for a false positive result).

Many of the genes located within these regions are of unknown function or did not have a role in muscle or bone development, which would make them unlikely candidate genes for clubfoot. Within the chromosome 3 region, the *CAND2* and *WNT7a* genes are involved in lower limb development, as *CAND2* is highly expressed in skeletal muscle and upregulated during the embryonic period of limb formation, while *WNT7a* has a role in dorsal patterning of vertebrate limbs. The temporal expression of *CAND2* and *WNT7a* is relevant because clubfoot most often develops during the second trimester of pregnancy; therefore, we are looking for a causative agent that may initiate its pathological effect earlier, but results in the deformity in later fetal development. Since these genes encode regulatory proteins, we could predict a delayed effect from the mutations as these defective proteins initiate the embryo on a developmental course that progresses as additional stresses are added to the developing limbs with increasing morphological complexity and size.

Therefore, we hypothesized the mutation frequencies for *CAND2* and *WNT7a* would be greater in patients with congenital idiopathic clubfoot than in controls.

Materials and Methods

We identified 256 patients with congenital idiopathic clubfoot without any other congenital abnormalities.

Patients with neuromuscular or other recognizable syndromes involving clubfoot were excluded. Seventy-five gender- and ethnically matched individuals without any foot deformities were recruited from our scoliosis pedigree database and were used as controls. All protocols were approved by the human subjects review board of the University of Iowa Hospitals and Clinics and St. Louis Children's Hospital and Shriners Hospital for Children in St. Louis. Informed consent was obtained from all participants.

Seven to 14 mL of whole blood was collected from each participant by venipuncture. DNA was extracted using a previously described protocol [10]. The concentration of each sample was approximately 20 ng/ μ L and the samples were stored at 4°C. The mRNA sequences for *CAND2* and *WNT7a* were obtained through a nucleotide search on the NCBI Web site (<http://www.ncbi.nih.gov>) and the complete cds were used. For *CAND2* (13 exons), the mRNA was 4229 bps long, and the accession number was NM_012298. For *WNT7a* (four exons), the mRNA was 4041 bps long, and the accession number was NM_004625.

We designed primers for each exon of *CAND2* and *WNT7a* using the Primer3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), and the forward and reverse primers were used to amplify each exon from the genomic DNA using the PCR protocol (Table 1). The primers were compared to the "htsg" database using BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to ensure the primers annealed only where they were designed to anneal. After the primers were obtained, they were diluted to 20 μ M and stored at -20°C.

The general protocol used for the PCR reactions with the 331 DNA samples and all the primer pairs was 5 μ L 10X Qiagen PCR Buffer, 1 μ L dNTP (10 mM each), 3 μ L forward primer (20 μ M), 3 μ L reverse primer (20 μ M), 0.5 μ L *Taq* DNA polymerase (5 U/ μ L), 10 μ L Q solution, 25 μ L dH₂O, and 2.5 μ L 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. Twenty μ L of the purified DNA products were sent to the University of Iowa DNA facility for sequencing.

After sequencing, the sequences for each exon were analyzed using both BLAT and BLAST searches (<http://genome.ucsc.edu> and <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Both the positive and negative strands of each sample were simultaneously inputted into a BLAT search, and the percent homology and the position of the sequence

Table 1. Primer sequences and product sizes for WNT7a and CAND2 exons

Gene	Position	Forward primer	Reverse primer	Size (bp)
WNT7a	Exon 1 + promoter	CGTCTCGCACACTTGAC	tgccaactttgtcgaaacac	382
WNT7a	Exon 2	tggcctaagtccatctct	ggtgtttgggtgagcacata	286
WNT7a	Exon 3.1	tgaccccaactgtctctc	GGGCATCCACAAAGACCT	247
WNT7a	Exon 3.2	CAAAGAGAAGCAAGGCCAGT	aagccattttgcagcatctc	226
WNT7a	Exon 4.1	tgacctgtctgtgctcttc	TAGTTGGGCGACTTCTCGAT	297
WNT7a	Exon 4.2	CCCACCTTCCTGAAGATCAA	GACATAGCAGCACCAGTGGA	258
WNT7a	Exon 4.3	TACAACACCCACCAGTACGC	TTGAGGGCTCTGAGAGATTTT	290
WNT7a	Exon 4.4	TTCTGCTGAGGAGGGTACTTTT	gcagtcactttgattgcag	297
CAND2	Exon 1 + promoter	GGGATTCTGAACCTGAGACTCTA	GCTTCGAGAGGGGAAGAAG	490
CAND2	Exon 2	ctgaggtaactgcccataatc	caggatgctccacacaag	235
CAND2	Exon 3	ggccattgagttgccataat	cattggctcacctaagctc	400
CAND2	Exon 4	tgcatagacagctgagtgctg	cagcatccccaccaacttc	244
CAND2	Exon 5	ctggccaaagcagtaaaat	aaggctgtgttcacctccac	294
CAND2	Exon 6	gacctgtctctgggagatgag	agtgagtggtgggttaggc	399
CAND2	Exon 7	ggcaggtgggtagtttctt	aactgggggtcaactcag	283
CAND2	Exon 8	Cggtggcctcataacctt	CAGCCGGGTGATCTCATT	477
CAND2	Exon 8.2	CTTGGGGATGACCTGGAG	GCAGGAAGCCTTCAGCAG	469
CAND2	Exon 8.3	GTCAGTGGCCCTGTGCTC	ACACGGCCAGTGCATAC	470
CAND2	Exon 8.4	GCTGAGGTGGGTCAGGTG	Atgctcagtgccaaactgc	465
CAND2	Exon 9	Ggagcctaagcaccttctga	Aacatcagcccaagctcaag	247
CAND2	Exon 10	Gactttgtgcttggctgtg	Acagtgagcatgagaggtg	286
CAND2	Exon 11	Gtggggtttgtagcagaagc	Cccgactatcccttggtaca	297
CAND2	Exon 12	Ccttgctctgcaccattt	Ggactccataggtgctccag	209
CAND2	Exon 13.1	Ggccaggctcaataaagga	CAACTGTTGGCAGCCTCAC	482
CAND2	Exon 13.2	GGCTTACAGTGCCTTCTCCA	ACATGAGGTCCAAGCCTGTC	475
CAND2	Exon 13.3	TTGGAAGATGATACGGGTTTCT	aactctgcctcctgggttc	357

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. We then searched the sequences of the control subjects at the locations polymorphisms were found in the patient population and observed whether there were similar changes. We used chi square analyses to compare the genotype frequencies of the clubfoot patients and controls to determine whether the nucleotide base changes were more common in the clubfoot patients than in the controls.

Results

We found no difference ($p > 0.05$) for either SNP in mutation frequencies between the clubfoot patients and controls. After screening CAND2 and WNT7a as candidate genes for association with congenital idiopathic clubfoot, we found in CAND2, by direct sequencing of PCR-amplified DNA from our clubfoot population, a G-to-A

SNP at position 3394 on exon 3 in chromosome 3 in 56% of the affected patient population and 47% of the control. The polymorphism caused a silent mutation and thus no change in the amino acid sequence of CAND2. After sequencing WNT7a, we found a G-to-T SNP at position 13896353 of exon 1 on chromosome 3 in 28% of clubfoot patients (16 of 56) as well as in 18% of the control subjects (nine of 50). The SNP was found in the non-coding region upstream of the start codon in exon 1. In addition to the exons, the promoter and splicing sites of CAND2 and WNT7a were screened, and no differences were found compared to controls.

Discussion

In the continued search for an understanding of the etiology of clubfoot, a genetic cause has always remained at the forefront of the established hypotheses. In twin studies, monozygotic twins have a higher concordance rate of clubfoot than dizygotic twins [15] and first-degree relatives of clubfoot patients have a 20-times higher probability of

also having clubfoot than the general population [14]. Most relevant to this study, many investigators have indicated the etiology of clubfoot may be caused by a single major gene [9, 12, 14]. The purpose of this study was to evaluate CAND2 and WNT7a as candidate genes in clubfoot patients and matched controls, in order to determine if mutations occurred more frequently in patients with congenital idiopathic clubfoot than controls.

Several limitations of our study design should be addressed. First, we selected candidate genes from a linkage Dietz et al. [5] reported had a LOD score that did not exceed three, which is the standard score mostly used for a significant linkage. However, we believed the linkage still warranted investigation because it was the first and only genome-wide linkage analysis conducted in clubfoot families and a LOD score greater than two reasonably encourages further analysis. In addition, this linkage contained CAND2 and WNT7a, which were very relevant genes with respect to early limb development. Second, we did not screen the non-coding (intronic) regions of CAND2 and WNT7a, and it is possible there may be a transcription binding site or enhancer region in the intronic sequence that could contain mutations. However, in genetic screens it is generally accepted not to evaluate the intronic regions given the excessive amount of DNA sequence information and the fact that the specific location of regulatory binding sites within the intronic regions are often unknown, as was the case in our genes. Third, we used relatively small patient and control populations, but 256 patients and 75 controls are generally sufficient for a preliminary screen (specifically when the phenotype is very well defined as in this population). However, it is possible we could not find some mutations that could cause a small proportion of clubfoot cases. Finally, since the predominant inheritance theories for clubfoot are that there is a single causative gene with a multigenetic susceptibility background, while we might miss a minor mutation that contributes to the susceptibility, we would expect to find the single causative mutation in the majority of the patients. The susceptibility background would consist of gene mutations that increase the likelihood of developing clubfoot in collaboration with other genetic and environmental factors, but would not alone directly cause clubfoot.

CAND2, also known as TIP120B, is a plausible candidate gene because it has high expressivity in skeletal muscle. Specifically, CAND2 is believed to be induced during embryogenesis and is localized only in muscle, whereas TIP120A has ubiquitous expression [1]. Also, CAND2 is a transcription factor that regulates myogenesis by suppressing the ubiquitination of myogenin. A mutation in CAND2 could disrupt early developmental myogenesis, which would be relevant to the development of clubfoot since the majority of clubfoot develops during the mid

trimester of pregnancy. However, since the SNP found in CAND2 created a silent mutation, CAND2 is most likely not a relevant candidate gene.

WNT7a is a plausible candidate gene because of its effect on cell fate, limb development, and temporal regulation. The WNT protein family has roles in cell proliferation, migration, polarity, and cell death. WNT7a is a secreted protein that stimulates the homeobox-containing gene *Lmx-1*, which together stimulate dorsal patterning in the vertebrate limb. It is plausible a mutation in WNT7a could affect the protein's ability to confer dorsal patterning thus disturbing normal axis development of the limb. In clubfoot, there is a decreased size of the muscles in the posterior compartment of the leg, therefore, abnormal expression or regulation of this gene may result in the deformity.

Several mutations in WNT7a apparently cause severe limb deformities and Fuhrmann syndrome, characterized by underdeveloped or absent arm and leg bones, but we did not find these mutations in clubfoot patients [13]. None of these mutations were found in our analysis, but we did identify a SNP upstream of the start codon. While the SNP in WNT7a was more common in clubfoot patients than in controls, it was still not statistically different. It is possible this SNP could be a susceptibility modifier rather than the single causative gene for clubfoot, and screening this SNP in a larger population may be warranted to definitively define the susceptibility status of this SNP. However, a recent study corroborated our finding that WNT7a is most likely not the major causative gene for clubfoot [8].

Based on our data of CAND2 and WNT7a, it is unlikely either of these genes are the major gene that causes clubfoot. The search for the major gene for congenital idiopathic clubfoot is of great importance because until we know the true mechanism behind clubfoot, we will never be able to treat the disease properly; rather, we will only be able to treat the terminal phenotype of the mechanism.

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