Published in final edited form as:

J Med Genet. 2016 April ; 53(4): 250–255. doi:10.1136/jmedgenet-2015-103505.

Deletions of 5' HOXC genes are associated with lower extremity malformations including clubfoot and vertical talus

David M. Alvarado[1], **Kevin McCall**[1], **Jacqueline T. Hecht**[4], **Matthew B. Dobbs**[1,5], and **Christina A. Gurnett**[1,2,3,*]

[1]Department of Orthopaedic Surgery, Washington University, St. Louis, MO, USA

[2]Department of Neurology, Washington University, St. Louis, MO, USA

[3]Department of Pediatrics, Washington University, St. Louis, MO, USA

[4]Department of Pediatrics, University of Texas Medical School, Houston Texas, USA

[5]Shriners Hospital for Children, St. Louis, MO, USA

Abstract

Background—Deletions of the *HoxC* gene cluster result in variable phenotypes in mice, but have been rarely described in humans. Here, we report chromosome 12q13.13 microdeletions ranging from 13-175 kb and involving the 5' *HOXC* genes in four families segregating congenital lower limb malformations, including clubfoot, vertical talus, and hip dysplasia.

Methods—Probands (N=253) with clubfoot or vertical talus were screened for point mutations and copy number variants (CNVs) using Multiplexed Direct Genomic Selection (MDiGS), a pooled BAC targeted capture approach. SNP genotyping included 1178 clubfoot or vertical talus probands and 1775 controls.

Results—The microdeletions share a minimal noncoding region overlap upstream of *HOXC13*, with variable phenotypes depending upon *HOXC13*, *HOXC12* or the *HOTAIR* lncRNA inclusion. SNP analysis revealed *HOXC11* p.Ser191Phe segregating with clubfoot in a small family and enrichment of *HOXC12* p.Asn176Lys in patients with clubfoot or vertical talus (rs189468720, p=0.0057, OR=3.8). Defects in limb morphogenesis include shortened and overlapping toes, as well as peroneus muscle hypoplasia. Finally, *HOXC* and *HOXD* gene expression is reduced in fibroblasts from a patient with a 5' *HOXC* deletion, consistent with prior studies demonstrating that dosage of lncRNAs alters expression of *HOXD* genes in *trans*.

Conclusions—Because *HOXD10* has been implicated in the etiology of congenital vertical talus, variation in its expression may contribute to the lower limb phenotypes occurring with 5'

*Corresponding Author: Christina A. Gurnett, MD PhD, Department of Neurology, Washington University School of Medicine, gurnettc@neuro.wustl.edu, 660 S Euclid Ave, St Louis, MO 63110, (314) 286-2789, FAX: (314) 286-2894.

COMPETING INTERESTS

None declared

CONTRIBUTORSHIP STATEMENT

DMA: conceived of the analysis, performed experiments, analyzed the data and wrote the manuscript; KM: performed experiments. JTH: collection of samples and provided genotype data; MBD collection of samples and clinical analysis; CAG: designed the study and supervised the research.

HOXC microdeletions. Identification of 5' *HOXC* microdeletions highlights the importance of transcriptional regulators in the etiology of severe lower limb malformations and will improve their diagnosis and management.

Keywords

Copy number; clubfoot; vertical talus

INTRODUCTION

Congenital vertical talus is a fixed dorsal dislocation of the talonavicular joint combined with contracture of the hindfoot that causes a rigid flatfoot deformity[1]. While commonly referred to as "rocker-bottom foot" because of its clinical appearance, diagnosis of congenital vertical talus requires radiographic confirmation. Talipes equinovarus (clubfoot) is a closely related disorder that may occur in families with vertical talus, or even in the same individual (i.e. clubfoot on the right foot and vertical talus on the left)[2 3]. Both clubfoot and vertical talus occur more frequently in males, with an approximate 2:1 male to female ratio[4]. Clubfoot has a birth prevalence of 1 in 1000 while 1 in 10,000 has been suggested for vertical talus[5], but may be an underestimate because of the difficulty in distinguishing from other more common and benign positional foot anomalies[1].

Vertical talus is associated with additional congenital anomalies in slightly more than half of all cases[3 5]. Syndromic causes of vertical talus include distal arthrogryposis and myelomeningocele, as well as trisomy 13 and trisomy 18[2 6 7]. Many of these disorders are associated with significant morbidity and mortality and require specialized surgical or neonatal intensive care in the newborn period; therefore genetic analysis may be essential for prenatal diagnosis to distinguish them from other causes of isolated vertical talus.

Congenital vertical talus has previously been associated with a single missense mutation (M391K) in *HOXD10* in two separate families[8 9]. However, the genetic cause of five other families with congenital vertical talus was not found, and *HOXD10* mutations were not found in sporadic cases of vertical talus[10]. While the genetic basis of clubfoot is also poorly understood, abnormalities in genes involved in early limb development including *PITX1* and *TBX4* are responsible for approximately 1-5% of familial clubfoot[11-15]. A deletion of *HOXC13* was also identified previously in one family with familial clubfoot[11]. Here, we describe chromosome 12q13 microdeletions involving the 5' *HOXC* genes in four families with vertical talus or clubfoot, and demonstrate trans-acting effects of this deletion on *HOXD* gene expression.

METHODS

Patient samples

The study protocol was approved by the Institutional Review Board of Washington University in St Louis and University of Texas at Houston and all subjects and/or parents gave informed consent. Blood and saliva samples were collected from probands and family members with lower limb malformations. The study included 1178 clubfoot or vertical talus

probands and 1775 controls (474 In-House and 1301 European Exome Aggregation Consortium [16]. All probands from Washington University in St Louis had either clubfoot or vertical talus. Most cases were isolated, but ~10% of the cases recruited from Washington University had additional musculoskeletal abnormalities, including tibial hemimelia, fibular hemimelia, or hip dysplasia. Affymetrix Genome-Wide Human SNP Array 6.0 was performed on 226 clubfoot and 27 congenital vertical talus probands and revealed only the 175 kb deletion in Family 1 as previously reported [11]. All samples had previously been screened for *PITX1* and *TBX4* point mutations, duplications and deletions. All families with vertical talus had *HOXD10* gene sequencing [9 10]. Patients were excluded from the study if they had multiple congenital anomalies or known causes of clubfoot or vertical talus. DNA was extracted using either the Roche DNA Isolation Kit for Mammalian Blood (Roche) or the Oragene Purifier for saliva (DNA Genotek). Magnetic resonance imaging was performed on a research basis as previously described[17].

MDiGS targeted resequencing

Probands (N=253) with clubfoot or vertical talus were screened for copy number variants (CNVs) using Multiplexed Direct Genomic Selection (MDiGS), a pooled BAC capture approach for targeted CNV detection[18]. Patient 1 was included as a positive control, as a 175kb deletion had previously been detected with Affymetrix 6.0 microarray[11]. Advantages of the MDiGS method are that the sequence data can be used to detect point mutations and for high resolution copy number analysis. Samples were individually indexed and hybridized to a biotinylated BAC bait RP11-578A18 (BACPAC Resources) in pools of 96 samples and post-capture samples were sequenced on a MiSeq Personal Sequencer (Illumina). The human chromosome 12 BAC RP11-578A18 encompasses a 184 kb region that includes *HOXC13, HOXC12, HOXC11, HOXC10* and *HOXC9* genes along with 108 kb non-coding region 5' of the *HOXC* gene cluster. Five RefSeq lncRNAs, including *HOTAIR*, were also located in the captured region. Copy number was determined using normalized read counts for each test sample compared to the average normalized read counts for all other co-captured samples and exact breakpoints were identified by gapped alignment of one-end anchored read pairs as previously described[18]. Copy number variants were validated by quantitative PCR using three PCR primers per CNV.

Restriction digest genotyping

The rs189468720/*HOXC12* SNP was genotyped in 625 clubfoot and 7 vertical talus cases and 474 controls using restriction digest (Washington University cohort) and in 336 clubfoot cases using TaqMan probes (University of Texas cohort). A 425 bp region containing rs189468720/*HOXC12* was PCR amplified (F-primer: GTGGAGGACGGCAAGGG, R-primer: CTAGCTCAGTCCTGTTCTGCC) from genomic DNA. PCR products were restriction digested with EcoNI (37C, 16 hrs.), which specifically cuts the rs189468720 alternate allele, producing 163 bp and 262 bp products.

Human skin fibroblasts

Human skin fibroblasts were cultured using previously described methods[19]. Skin was obtained during clubfoot surgery from patient 1 (*HOXC* deletion) and two reference samples without *HOXC* deletions.

Gene expression analysis

Total RNA was extracted from confluent human skin fibroblasts, 1 T75 flask per sample/replicate, using Trizol (Invitrogen) recommended protocol, DNase-digested with TURBO DNase and further purified using RNeasy (Qiagen). Total RNA was run on an agarose gel to inspect for degradation and quantified on a Nanodrop 2000. 1 μg RNA was used for SuperScript II reverse transcription with random hexamers.

Gene expression was assayed on an Mx3005P (Stratagene) using iTaq Universal SYBR Green Supermix (BioRad), 40 cycles followed by a melt curve analysis. All cDNA samples were run in triplicate with mean Ct values compared to a beta-actin (ACTB) reference for ddCt calculation. Relative expression ratios represent mean ratios for 3 deletion biological replicates compared to 2 controls, 3 biological replicates each. Error bars represent standard deviation across all case/control comparisons and p values were calculated using a Student's T-test for each gene compared to 5 housekeeping controls (18s rRNA, PUM1, GAPDH, HPRT1 and SDHA).

RESULTS

To investigate the role of *HOXC* gene cluster variation in human congenital lower limb malformations, we captured and sequenced[18] a 184 kb region including 5' *HOXC* genes (*HOXC13, HOXC12, HOXC11, HOXC10* and *HOXC9*) and lncRNAs (*HOTAIR, HOXC13-AS, HOXC-AS3, HOXC-AS2* and *HOXC-AS1*) in 226 clubfoot and 27 congenital vertical talus probands. Copy number analysis revealed three small deletions ranging in size from 13-52 kb, as well as our previously reported 175 kb chr12:54165001-54335668 deletion in patient 1[11] (Figure 1). While these four microdeletions overlap within a 5.3 kb noncoding region (hg19, chr12:54311194-54316500) located 16 kb 5' of the *HOXC* gene cluster, there are no genes within the overlapping region. The 175kb deletion in family 1 contains only exon 2 of *HOXC13* and *HOXC13-AS* lncRNA. The 13 kb microdeletion in family 2 contains no genes and is located entirely 5' of the *HOXC* gene cluster but has been previously reported in 2/2504 healthy individuals in the Database of Genomic Variants (DGV) [20]. There are no reported microdeletions involving *HOXC13-AS* and *HOXC13* in DGV. The nearly identically sized 50 kb deletions in families 3 and 4 share the same distal breakpoint but have different proximal breakpoints, and include *HOXC13, HOXC12* and part of *HOTAIR* lncRNA. The microdeletions segregate with lower limb malformations in each family and were fully penetrant with variable expressivity (Figure 2).

Sequence analysis of the MDiGS data revealed two rare variants predicted to be damaging by SIFT [21]. A rare *HOXC11* p.Ser191Phe missense variant (EU-MAF=0.0002) that completely segregates with clubfoot in six affected individuals of family 5 (Figure 3). We also identified a rare *HOXC12* p.Asn176Lys (rs189468720) missense variant in 5/210 (MAF=0.0119) Caucasian clubfoot and vertical talus cases compared to 0/321 in-house controls and 4/1301 European (MAF=0.0015) individuals from the Exome Aggregation Consortium (ExAC) (p-value=0.0016, OR=9.84)[16]. After genotyping the rs189468720 variant in additional 961 clubfoot and 7 vertical talus cases and 153 controls, we confirmed a weak association of rs189468720 with lower limb malformations (combined cases = 15/1178, controls = 6/1775, p-value = 0.0057, OR=3.8).

Clinical features of patients with HOXC deletions

The largest deletion identified was 175 kb that was present in four affected members of family 1 and involved only *HOXC13* and *HOXC13-AS* lncRNA. Three members of this family had severe treatment-resistant clubfoot that required multiple surgeries (Figure 4A and 4B) (Supplementary Table 1), and one had hammertoes that required surgical treatment (Figure 4C). One individual had nail hypoplasia involving all fingers but not toes (Figure 4D).

The smallest 13 kb intergenic microdeletion, also reported in DGV, occurred in the proband of family 2 who had right-sided clubfoot and fibular hemimelia and post-axial hypodactyly with absent fourth and fifth toes on the right foot. The microdeletion also occurred in his father who had mild intoeing and unilateral 2-3 toe syndactyly but no other limb abnormality.

Congenital vertical talus occurred with complete penetrance in all nine affected individuals from families 3 and 4 who harbored the similar, but not-identical, 50kb microdeletions containing *HOXC13, HOXC12*, and part of *HOTAIR* lncRNA (Figure 4 E-K) (Supplementary Table 1). Individuals from both families had recurrent deformity requiring additional casting and surgery. In addition, hip dysplasia and hip muscle weakness were present in childhood, and severe arthritis of the hip and knee were present in older individuals with the microdeletion. One individual was noted to have moderate bilateral adducted thumbs in infancy that resolved with time. Brittle toenails were noted in two individuals.

Muscle volume is concordantly reduced in patients with HOXC deletions

Magnetic resonance imaging (MRI) of the lower limbs was performed to characterize the limb malformation as some morphological abnormalities can only be detected with imaging[22]. MRIs were obtained at age 17 (1-001) and age 64 (1-004) from two affected members of family 1 who had bilateral clubfoot that was more severe on the right, the MRI revealed smaller muscle compartments in the calf, with the peroneus muscles in the lateral compartment most severely affected and partially replaced with fat (Figure 5 A-B). Peroneus muscle hypoplasia was concordant in both individuals and was more prominent in the more severely affected right legs. Globally reduced muscle volume of the affected limb was also observed on MRI obtained from two siblings with unilateral clubfoot in family 5 (right limb, Figure 5C; left limb Figure 5D).

Cis and trans effects of *HOXC* microdeletions

To determine if deletion of 5' *HOXC* genes and upstream regulatory regions affect expression levels of downstream genes, we performed qPCR on human skin fibroblasts from the family 1 proband with the 175 kb microdeletion and two reference samples without *HOXC* deletions. The deletion removes the first exon of *HOXC13* and its transcript was reduced approximately 50% compared to controls (Figure 6). Although *HOXC12* is not located within the deletion, its transcript was also decreased. The expression of *HOTAIR* lncRNA, which is outside the deleted interval, was unaffected; however, lncRNAs *HOXC-AS2* and *HOXC-AS3*, also outside the deletion interval, were decreased. Because *HOXC*

lncRNAs regulate *HOXD* gene expression[23] and *HOXD*10 mutations have previously been identified in patients with vertical talus[8 9], we also examined the expression levels of *HOXD* genes in human skin fibroblasts. The expression of four *HOXD* genes, *HOXD13, HOXD12, HOXD11,* and *HOXD10* were significantly reduced compared to controls.

DISCUSSION

To interrogate the role of rare *HOXC* gene cluster variation in congenital lower limb malformations we used MDiGS, a novel multiplexed targeted capture approach that is useful for both highly accurate CNV and SNP/INDEL discovery in large genomic regions[18]. Four unrelated families with congenital vertical talus and clubfoot were found to have small microdeletions of 5' *HOXC* genes. While vertical talus and clubfoot associated with 5'*HOXC* microdeletions is nearly completely penetrant and more difficult to treat clinically, the prognosis is much better than many other more common causes of vertical talus, such as trisomy 18[2], and these may therefore be clinically important to identify. Notably, the 5' *HOXC* gene microdeletions we detected are small (<200kb) and may be difficult to detect with routine chromosomal microarray. In fact, only one of our four deletions was detected by chromosomal microarray (Affymetrix 6.0), and the other three required higher resolution MDiGS. With our current methods, 5' *HOXC* microdeletions were detected in 2 out of 6 previously described autosomal dominant isolated vertical talus families[9], and we anticipate that improvements in technology will allow for the identification of even smaller microdeletions that may be responsible for additional cases.

Earlier studies in mice demonstrated expression of 5' *HoxC* genes in the developing hindlimb but not the forelimb, suggesting that they might play an important role in hindlimb morphogenesis[24]. The phenotypes of our patients with 5' *HOXC* deletions are consistent with hindlimb expression, as none of our patients had hand malformations with the exception of one patient with fingernail hypoplasia. Despite their unique expression pattern, however, it was believed that 5' *HoxC* genes were unnecessary for limb development because loss of the entire *HoxC* gene cluster or loss of individual 5' *HoxC* genes (*Hoxc9, Hoxc10, Hoxc13*) did not result in lower limb skeletal abnormalities in mice[25]. While loss of the entire *HOXC* gene cluster has been described in three individuals with large chromosome 12 deletions (>1 Mb) and cognitive impairment, the loss of additional genes precludes the assignment of *HOXC* gene effects. Skeletal abnormalities described previously in these patients include ulnar deviation of hands, flexion deformities of fourth and fifth fingers and contractures of Achilles tendons[26], severe kyphoscoliosis, ulnar deviation of the hands and finger flexion contractures[27], and arthrogryposis with valgus ankle position and pectus excavatum[28]. Microdeletions of 5' *HOXC* genes as described here have not been reported by other investigators and none are annotated in DGV or the DECIPHER database[29]. Interestingly, the reciprocal microdeletion in which all *HOXC* genes except *HOXC13*, *HOXC12* and *HOTAIR* are deleted was not associated with any skeletal defects or limb contractures [30], suggesting that the limb malformations in our families are specifically related to abnormalities in one of these genes. The smallest 13 kb intergenic microdeletion that we identified in one family had been reported in 2 out of 2504 controls in DGV, suggesting that it may be either a benign variant or a modifier.

Because there are strong global regulators controlling the coordinated expression of *Hox* genes and positional effects of deletions can alter the colinear expression of *HoxC* genes[31], it is difficult to ascribe functional effects of single genes within the *HOXC* locus. Certainly, the incompletely penetrant nail hypoplasia seen in one of our cases is consistent with loss of *HOXC13* within the deleted interval, although pure hair and nail ectodermal dysplasia due to *HOXC13* mutations has only been described as an autosomal recessive condition[32]. The association of single missense mutations in *HOXC11* and *HOXC12* with isolated clubfoot and vertical talus also suggests a possible role for single 5' *HOXC* gene alterations in their pathogenesis, although additional studies are needed for confirmation.

Our data also support an intriguing possibility that modification of the *HOXC* locus on chromosome 12 *trans*-regulates expression of *HOXD* genes on chromosome 2. An indirect effect is plausible because mutations in *HOXD10* cause congenital vertical talus[8 9]. The 5' *HOXC* region contains several lncRNAs including *HOXC13-AS, HOXC-AS2*, *HOXC-AS3* transcripts, and *HOTAIR,* the latter of which has been shown to be a repressor of *HoxD* gene expression in mice[23 33]. Our study likewise demonstrates in human disease fibroblasts from a patient with a *HOXC* microdeletion, altered lncRNA expression and downregulation of *HOXD* gene expression. Overall, this suggests that *HOXC* locus differential *trans*-regulation of the *HOXD* genes may be an important factor in some human lower limb malformations.

The reduced limb muscle mass and peroneus muscle hypoplasia observed in two patients with 5' *HOXC* gene microdeletions was similar to the morphological abnormalities identified in humans and mice with clubfoot secondary to *PITX1* deletions[12 13]. As a consequence of these morphological abnormalities[22], patients with 5' *HOXC* deletions are more likely to have difficult to treat vertical talus and clubfoot and additional skeletal comorbidities, such as hip dysplasia. More severe clinical phenotypes have also been described in clubfoot patients with *TBX4* duplications[15 34] and *PITX1* mutations[12 13]. Because *Pitx1* acts upstream of *Tbx4* and *Hox* genes during hindlimb specification[35] and binds directly to regulatory elements near *Hoxc10* and *Hoxc11*[36], our results support a critical role for hindlimb transcriptional regulators in clubfoot and vertical talus pathogenesis.

Most importantly, this study provides clinically relevant information regarding the genetic basis of clubfoot and vertical talus that will be immediately applicable to the diagnosis and genetic counseling of patients with these disorders. Because *HOXC* microdeletions are associated with increased risk of severe treatment resistance and hip dysplasia, accurate molecular diagnosis will ultimately translate into personalized and improved management of patients with lower limb malformations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We thank the patients and their families for their role in this work. We thank the Genome Technology Access Center in the Department of Genetics at Washington University School of Medicine for providing Illumina MiSeq Personal Sequencer support.

FUNDING

This work was supported by Shriners Hospital for Children, the Children's Discovery of St Louis Children's Hospital and Washington University, and the Eunice Kennedy Shriver National Institute of Child Health and Human Development grant number R03HD068649.

References

1. Lamy L, Weissman L. Congenital convex pes valgus. J Bone Joint Surg Am. 1939; 21:79.
2. Merrill LJ, Gurnett CA, Connolly AM, Pestronk A, Dobbs MB. Skeletal muscle abnormalities and genetic factors related to vertical talus. Clin Orthop Relat Res. 2011; 469(4):1167–74. doi: 10.1007/s11999-010-1475-5. [PubMed: 20645034]
3. Ogata K, Schoenecker PL, Sheridan J. Congenital vertical talus and its familial occurrence: an analysis of 36 patients. Clin Orthop Relat Res. 1979; (139):128–32. [PubMed: 455829]
4. Coleman SS, Stelling FH 3rd, Jarrett J. Pathomechanics and treatment of congenital vertical talus. Clin Orthop Relat Res. 1970; 70:62–72. [PubMed: 4910900]
5. Jacobsen ST, Crawford AH. Congenital vertical talus. J Pediatr Orthop. 1983; 3(3):306–10. [PubMed: 6874927]
6. Aroojis AJ, King MM, Donohoe M, Riddle EC, Kumar SJ. Congenital vertical talus in arthrogryposis and other contractural syndromes. Clin Orthop Relat Res. 2005; (434):26–32. [PubMed: 15864028]
7. Westcott MA, Dynes MC, Remer EM, Donaldson JS, Dias LS. Congenital and acquired orthopedic abnormalities in patients with myelomeningocele. Radiographics. 1992; 12(6):1155–73. [PubMed: 1439018]
8. Shrimpton AE, Levinsohn EM, Yozawitz JM, Packard DS Jr. Cady RB, Middleton FA, Persico AM, Hootnick DR. A HOX gene mutation in a family with isolated congenital vertical talus and Charcot-Marie-Tooth disease. Am J Hum Genet. 2004; 75(1):92–6. [PubMed: 15146389]
9. Dobbs MB, Gurnett CA, Pierce B, Exner GU, Robarge J, Morcuende JA, Cole WG, Templeton PA, Foster B, Bowcock AM. HOXD10 M319K mutation in a family with isolated congenital vertical talus. J Orthop Res. 2006; 24(3):448–53. [PubMed: 16450407]
10. 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
11. Alvarado DM, Buchan JG, Frick SL, Herzenberg JE, Dobbs MB, Gurnett CA. Copy number analysis of 413 isolated talipes equinovarus patients suggests role for transcriptional regulators of early limb development. Eur J Hum Genet. 2013; 21(4):373–80. [PubMed: 22892537]
12. Alvarado DM, McCall K, Aferol H, Silva MJ, Garbow JR, Spees WM, Patel T, Siegel M, Dobbs MB, Gurnett CA. Pitx1 haploinsufficiency causes clubfoot in humans and a clubfoot-like phenotype in mice. Hum Mol Genet. 2011; 20(20):3943–52. [PubMed: 21775501]
13. Gurnett CA, Alaee F, Kruse LM, Desruisseau DM, Hecht JT, Wise CA, Bowcock AM, Dobbs MB. Asymmetric lower-limb malformations in individuals with homeobox PITX1 gene mutation. Am J Hum Genet. 2008; 83(5):616–22. [PubMed: 18950742]
14. Alvarado DM, Aferol H, McCall K, Huang JB, Techy M, Buchan J, Cady J, Gonzales PR, Dobbs MB, Gurnett CA. Familial isolated clubfoot is associated with recurrent chromosome 17q23.1q23.2 microduplications containing TBX4. Am J Hum Genet. 2010; 87(1):154–60. [PubMed: 20598276]
15. Lu W, Bacino CA, Richards BS, Alvarez C, Vandermeer JE, Vella M, Ahituv N, Sikka N, Dietz FR, Blanton SH, Hecht JT. Studies of TBX4 and chromosome 17q23.1q23.2: An uncommon cause of nonsyndromic clubfoot. Am J Med Genet A. 2012; 158A(7):1620–7. [PubMed: 22678995]

16. Exome Aggregation Consortium. (ExAC); Cambridge, MA: (URL:http://exac.broadinstitute.org) [accessed July 20, 2015]
17. Kruse L, Gurnett CA, Hootnick D, Dobbs MB. Magnetic resonance angiography in clubfoot and vertical talus: a feasibility study. Clin Orthop Relat Res. 2009; 467(5):1250–5. [PubMed: 19127394]
18. Alvarado DM, Yang P, Druley TE, Lovett M, Gurnett CA. Multiplexed direct genomic selection (MDiGS): a pooled BAC capture approach for highly accurate CNV and SNP/INDEL detection. Nucleic Acids Res. 2014; 42(10):e82. [PubMed: 24682816]
19. Huschtscha LI, Napier CE, Noble JR, Bower K, Au AY, Campbell HG, Braithwaite AW, Reddel RR. Enhanced isolation of fibroblasts from human skin explants. BioTechniques. 2012; 53(4):239–44. doi: 10.2144/0000113939. [PubMed: 23046507]
20. MacDonald JR, Ziman R, Yuen RK, Feuk L, Scherer SW. The Database of Genomic Variants: a curated collection of structural variation in the human genome. Nucleic Acids Res. 2014; 42(Database issue):D986–92. doi: 10.1093/nar/gkt958. [PubMed: 24174537]
21. Ng PC, Henikoff S. Predicting deleterious amino acid substitutions. Genome Res. 2001; 11(5): 863–74. doi: 10.1101/gr.176601. [PubMed: 11337480]
22. Moon DK, Gurnett CA, Aferol H, Siegel MJ, Commean PK, Dobbs MB. Soft-tissue abnormalities associated with treatment-resistant and treatment-responsive clubfoot: findings of MRI analysis. J Bone Joint Surg Am. 2014; 96(15):1249–56. doi: 10.2106/JBJS.M.01257. [PubMed: 25100771]
23. Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Brugmann SA, Goodnough LH, Helms JA, Farnham PJ, Segal E, Chang HY. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell. 2007; 129(7):1311–23. [PubMed: 17604720]
24. Peterson RL, Papenbrock T, Davda MM, Awgulewitsch A. The murine Hoxc cluster contains five neighboring AbdB-related Hox genes that show unique spatially coordinated expression in posterior embryonic subregions. Mech Dev. 1994; 47(3):253–60. [PubMed: 7848872]
25. Suemori H, Noguchi S, Hox C. cluster genes are dispensable for overall body plan of mouse embryonic development. Dev Biol. 2000; 220(2):333–42. doi: 10.1006/dbio.2000.9651. [PubMed: 10753520]
26. Hancarova M, Simandlova M, Drabova J, Petrak B, Koudova M, Havlovicova M, Sedlacek Z. Chromosome 12q13.13 deletions involving the HOXC gene cluster: phenotype and candidate genes. Eur J Med Genet. 2012; 56(3):171–3. [PubMed: 23274590]
27. Okamoto N, Tamura D, Nishimura G, Shimojima K, Yamamoto T. Submicroscopic deletion of 12q13 including HOXC gene cluster with skeletal anomalies and global developmental delay. Am J Med Genet A. 2011; 155A(12):2997–3001. [PubMed: 22069146]
28. Jonsson DI, Ludvigsson P, Aradhya S, Sigurdardottir S, Steinarsdottir M, Hauksdottir H, Jonsson JJ. A de novo 1.13 Mb microdeletion in 12q13.13 associated with congenital distal arthrogryposis, intellectual disability and mild dysmorphism. Eur J Med Genet. 2012; 55(6-7):437–40. [PubMed: 22534424]
29. [accessed Aug 11, 2015] DECIPHER v9.0. https://decipher.sanger.ac.uk
30. Peterson JF, Hartman J, Ghaloul-Gonzalez L, Surti U, Hu J. Absence of skeletal anomalies in siblings with a maternally inherited 12q13.13-q13.2 microdeletion partially involving the HOXC gene cluster. Am J Med Genet A. 2013; 164A(3):810–4. [PubMed: 24443387]
31. Herault Y, Beckers J, Gerard M, Duboule D. Hox gene expression in limbs: colinearity by opposite regulatory controls. Dev Biol. 1999; 208(1):157–65. doi: 10.1006/dbio.1998.9179. [PubMed: 10075849]
32. Lin Z, Chen Q, Shi L, Lee M, Giehl KA, Tang Z, Wang H, Zhang J, Yin J, Wu L, Xiao R, Liu X, Dai L, Zhu X, Li R, Betz RC, Zhang X, Yang Y. Loss-of-function mutations in HOXC13 cause pure hair and nail ectodermal dysplasia. Am J Hum Genet. 2012; 91(5):906–11. doi: 10.1016/j.ajhg.2012.08.029. [PubMed: 23063621]
33. Li L, Liu B, Wapinski OL, Tsai MC, Qu K, Zhang J, Carlson JC, Lin M, Fang F, Gupta RA, Helms JA, Chang HY. Targeted disruption of Hotair leads to homeotic transformation and gene derepression. Cell reports. 2013; 5(1):3–12. doi: 10.1016/j.celrep.2013.09.003. [PubMed: 24075995]

34. Peterson JF, Ghaloul-Gonzalez L, Madan-Khetarpal S, Hartman J, Surti U, Rajkovic A, Yatsenko SA. Familial microduplication of 17q23.1-q23.2 involving TBX4 is associated with congenital clubfoot and reduced penetrance in females. Am J Med Genet A. 2014; 164A(2):364–9. [PubMed: 24592505]

35. Logan M, Tabin CJ. Role of Pitx1 upstream of Tbx4 in specification of hindlimb identity. Science. 1999; 283(5408):1736–9. [PubMed: 10073939]

36. Infante CR, Park S, Mihala AG, Kingsley DM, Menke DB. Pitx1 broadly associates with limb enhancers and is enriched on hindlimb cis-regulatory elements. Dev Biol. 2013; 374(1):234–44. doi: 10.1016/j.ydbio.2012.11.017. [PubMed: 23201014]

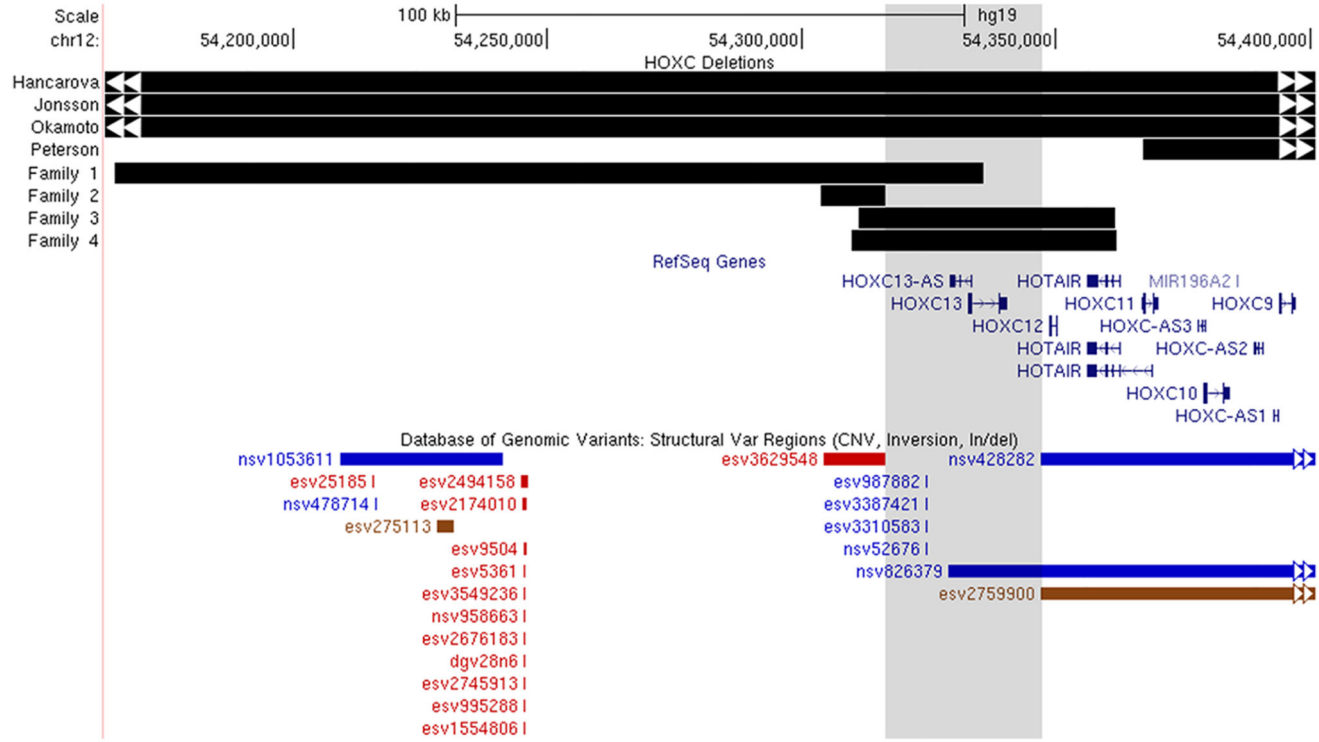

Figure 1. 5' *HOXC* gene microdeletions identified in clubfoot and vertical talus patients

Four partially overlapping deletions involving the 5' *HOXC* genes and upstream regulatory region were identified in clubfoot and vertical talus probands.

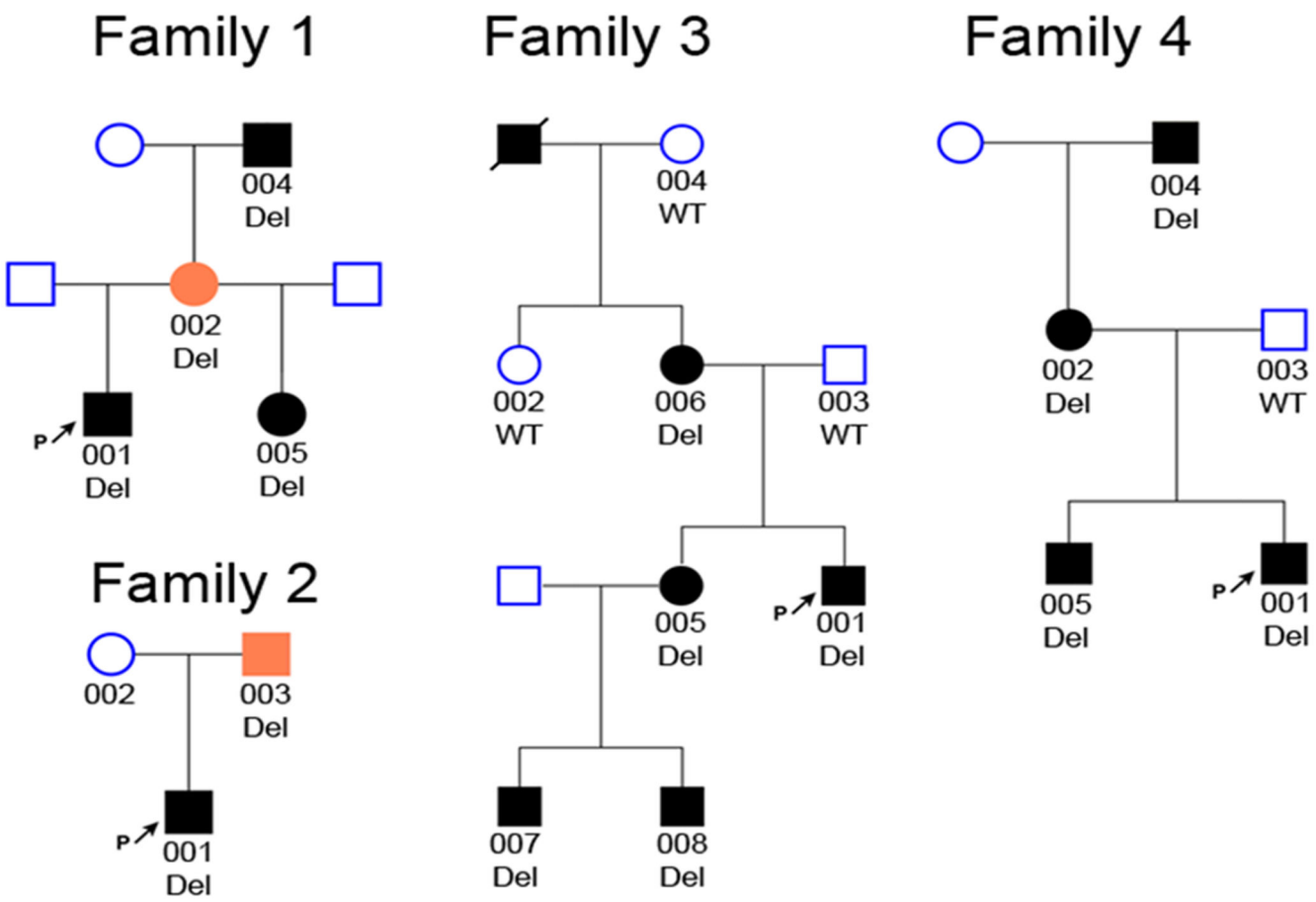

Figure 2. Segregation of 5' *HOXC* microdeletions with vertical talus and clubfoot

Black circles and squares indicate clubfoot in families 1 and 2 and vertical talus in families 3 and 4. Gray indicates syndactyly (2-003) and hammertoes (1-002). Del indicates deletion and WT indicates normal copy number.

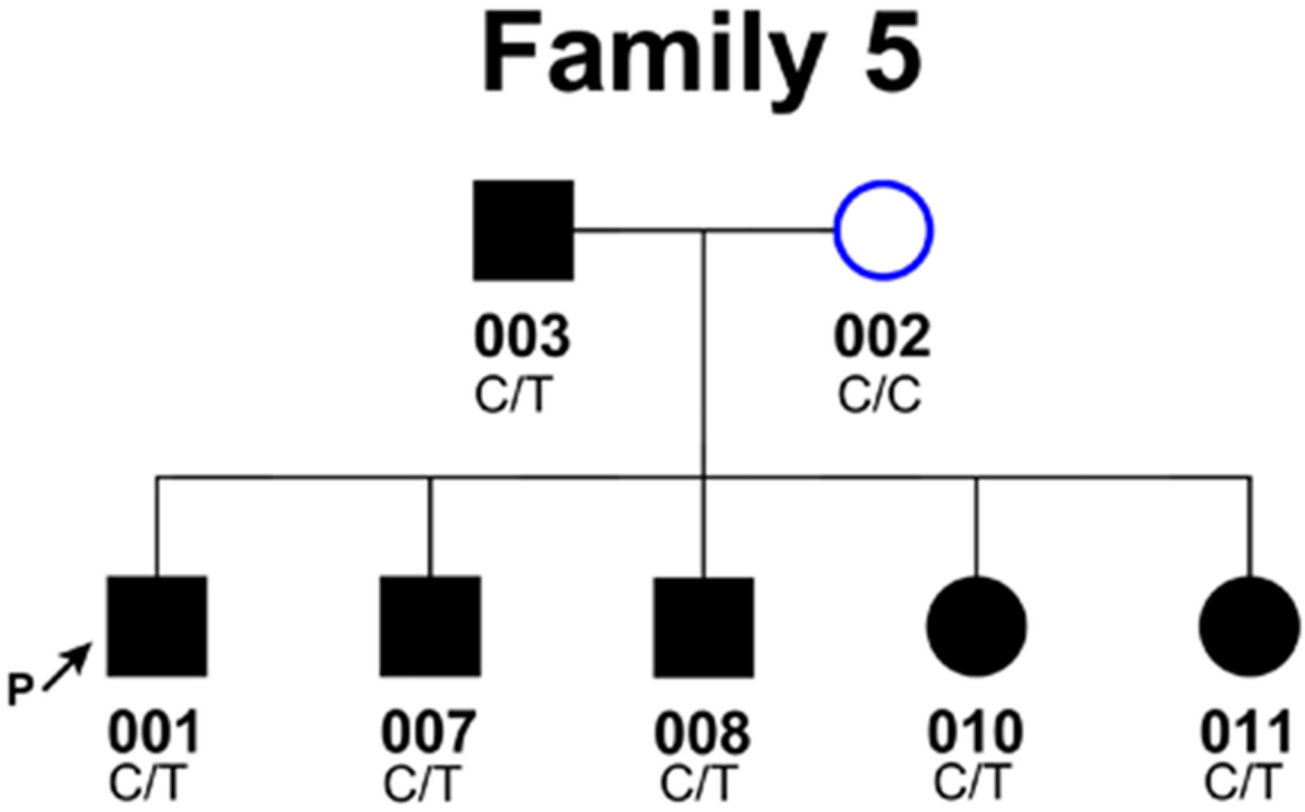

Figure 3. Segregation of *HOXC11* p.Ser191Phe with clubfoot

The *HOXC11* p.Ser191Phe missense variant completely segregates with clubfoot in six affected individuals in family 5.

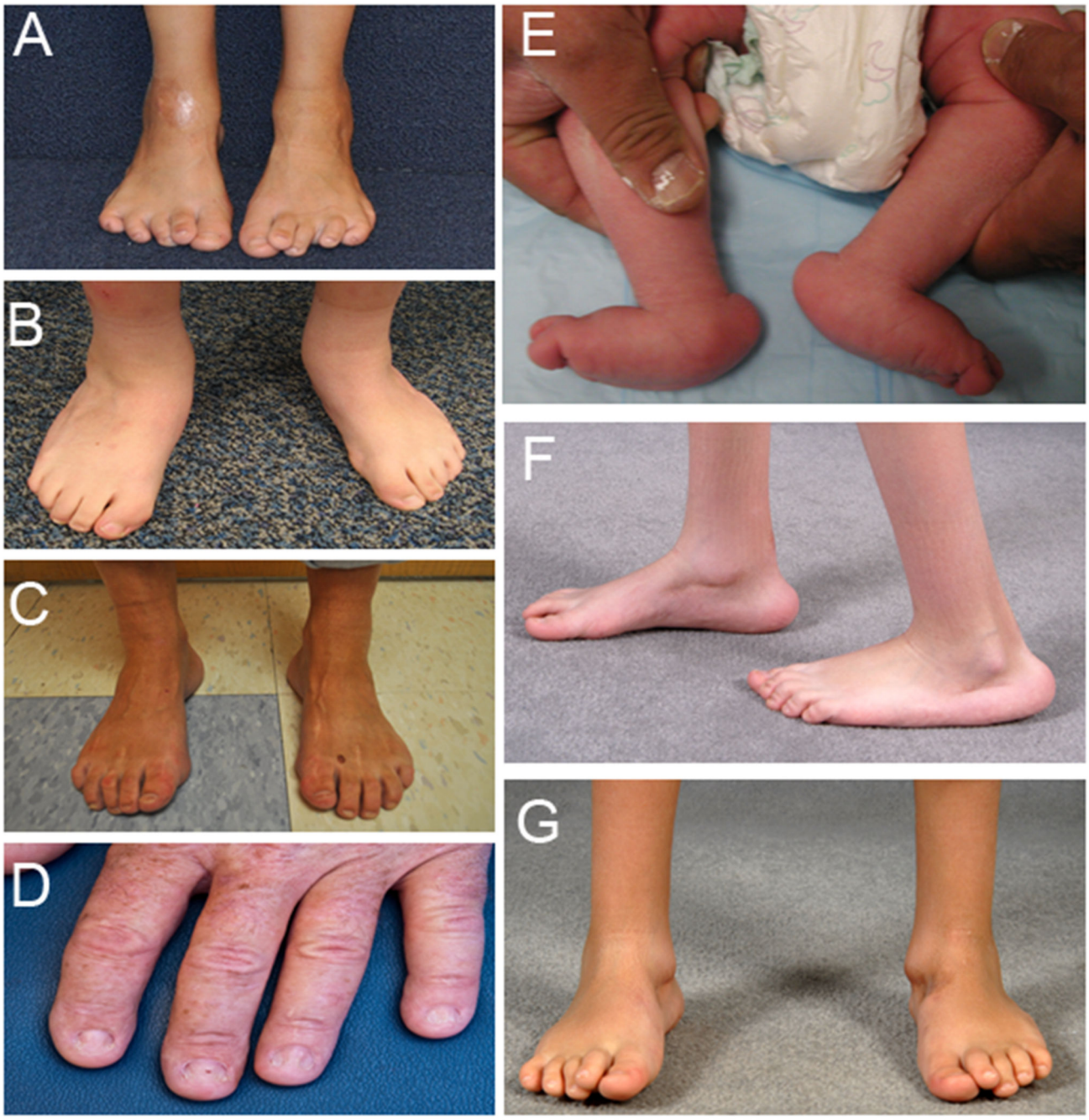

Figure 4. Clinical features of patients with 5' *HOXC* microdeletions

Treated clubfoot in (A) proband (1-001) and (B) sibling (1-005) with 175 kb 5' *HOXC* gene deletion. Note overlapping toes. (C) Surgically treated hammertoes (1-002) and (D) fingernail hypoplasia (1-004). Congenital vertical talus with (E) rockerbottom appearance (3-007) before treatment and (F-G) persistent flatfoot (3-007) and overlapping toes (3-008) after treatment. Treated congenital vertical talus with (H overlapping toes (4-001), (I) flatfoot and shortened 4^{th} and 5^{th} digits (4-004) and (J-K) hypoplastic 4^{th} digit (4-005).

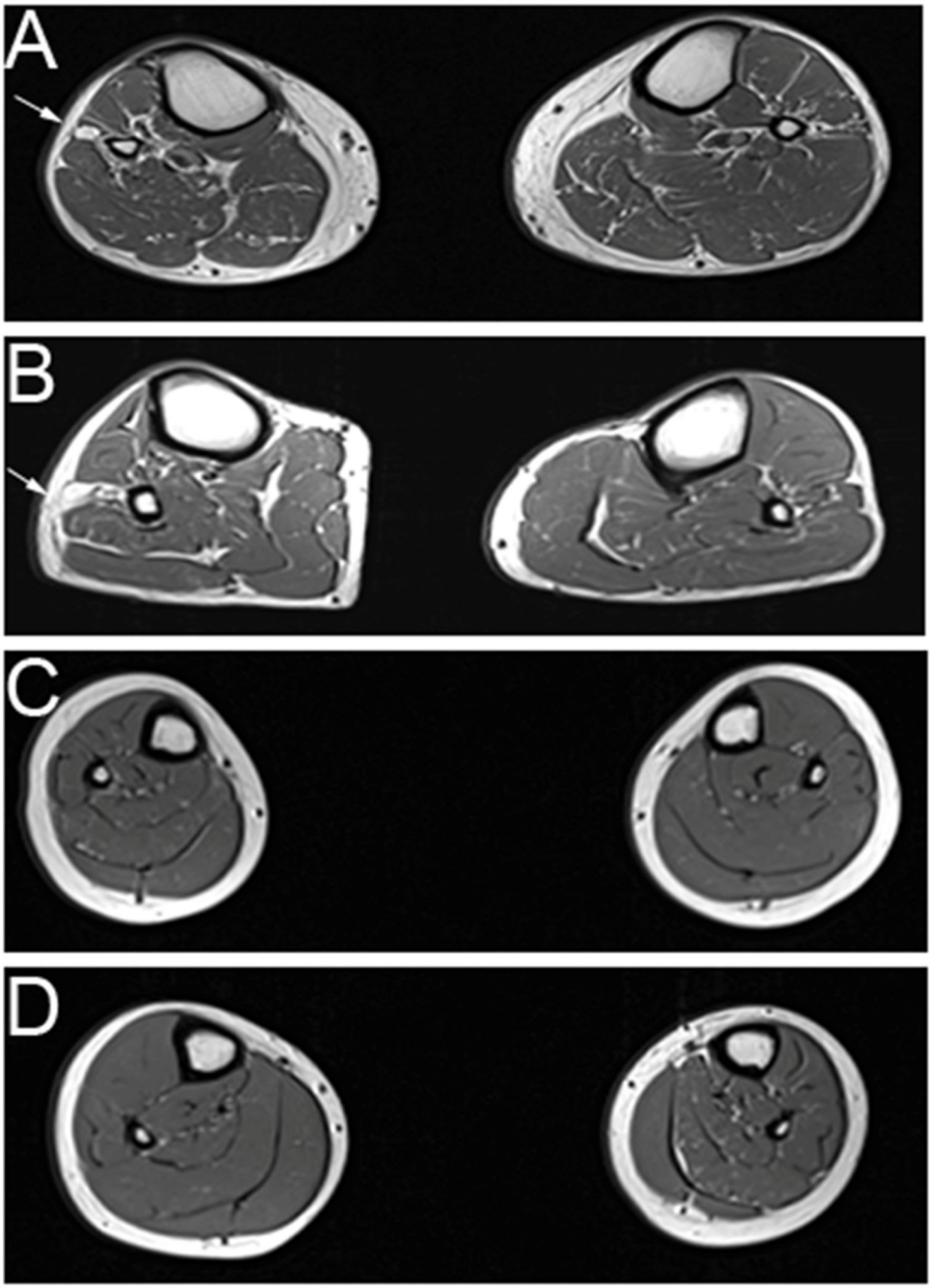

Figure 5. Magnetic resonance images show reduced muscle volume in the calf of patients with clubfoot and 5' HOXC gene deletions

Lateral compartment peroneus muscles are most severely affected and replaced with fat (arrows) in the more severely affected limb (left) in (A) 1-001 and (B) 1-004. Globally reduced muscle volume in the unilateral affected clubfoot limbs of siblings with *HOXC11* p.Ser191Phe mutations (C) 5-007 (left leg) and (D) 5-008 (right leg).

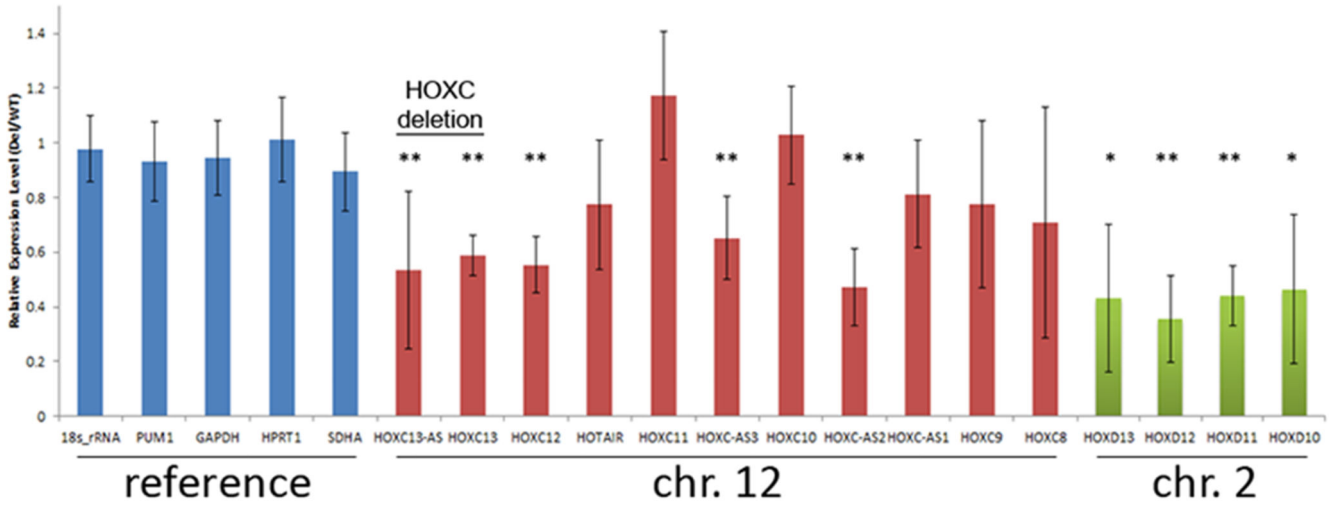

Figure 6. *HOXC* and *HOXD* gene expression is down-regulated in skin fibroblasts of patient with 5' *HOXC* gene deletion

HOXC and HOXD gene expression in human skin fibroblasts from clubfoot patient 1-001 with 175 kb *HOXC* deletion (*HOXC13* and *HOXC13-AS* deleted) compared to control fibroblasts. *P<0.05, **P<0.005.