Asymmetric Lower-Limb Malformations in Individuals with Homeobox *PITX1* Gene Mutation

Christina A. Gurnett,[1,2,3,*] Farhang Alaee,[1] Lisa M. Kruse,[1] David M. Desruisseau,[1] Jacqueline T. Hecht,[5] Carol A. Wise,[6] Anne M. Bowcock,[4] and Matthew B. Dobbs[1,7]

Clubfoot is one of the most common severe musculoskeletal birth defects, with a worldwide incidence of 1 in 1000 live births. In the present study, we describe a five-generation family with asymmetric right-sided predominant idiopathic clubfoot segregating as an autosomal-dominant condition with incomplete penetrance. Other lower-limb malformations, including patellar hypoplasia, oblique talus, tibial hemimelia, developmental hip dysplasia, and preaxial polydactyly, were also present in some family members. Genome-wide linkage analysis with Affymetrix GeneChip Mapping 10K mapping data from 13 members of this family revealed a multipoint LOD_{max} of 3.31 on chromosome 5q31. A single missense mutation (c.388G→A) was identified in *PITX1*, a bicoid-related homeodomain transcription factor critical for hindlimb development, and segregated with disease in this family. The *PITX1* E130K mutation is located in the highly conserved homeodomain and reduces the ability of PITX1 to transactivate a luciferase reporter. The *PITX1* E130K mutation also suppresses wild-type PITX1 activity in a dose-dependent manner, suggesting dominant-negative effects on transcription. The propensity for right-sided involvement in tibial hemimelia and clubfoot suggests that *PITX1*, or pathways involving *PITX1*, may be involved in their etiology. Implication of a gene involved in early limb development in clubfoot pathogenesis also suggests additional pathways for future investigations of idiopathic clubfoot etiology in humans.

Several congenital limb malformations exclusively affect the lower limb, including developmental hip dysplasia, proximal focal femoral deficiency, tibial and fibular hemimelia, and clubfoot (talipes equinovarus). Clubfoot is one of the most common serious congenital musculoskeletal anomalies with a worldwide incidence of 1 in 1000 live births.[1] Approximately 80% of clubfeet occur as isolated birth defects and are considered idiopathic.[2] Genetic factors play a role in the etiology of clubfoot, given that nearly 25% of all cases are familial.[3] Additional evidence for a genetic etiology is provided by differences in clubfoot prevalence across ethnic populations with the lowest prevalence in Chinese (0.39 cases per 1,000 live births) and the highest in Hawaiians and Maoris (7 per 1,000).[4,5] Males are more frequently affected (2:1 male to female ratio), and such a finding is consistent across ethnic groups.[4] Clubfoot is bilateral in approximately 50% of all cases, and the right foot is more often affected in unilateral cases.[3]

Limb patterning and growth are carefully regulated through a complex network of transcription-factor and signaling-molecule expression.[6,7] Two transcription-factor genes, *Pitx1* (MIM 602149) and *Tbx4* (MIM 601719), are expressed predominantly in the developing hindlimb and are only minimally expressed in the forelimb,[8–10] suggesting that they may be important regulators of hindlimb identity. In support of this, studies have shown that misexpression of *Pitx1* in the developing chick wing bud changes the morphology and digit number such that the limb more resembles a leg.[11,12] Likewise, loss of *Pitx1* expression in the developing mouse causes the hindlimb to assume morphology and growth features of the corresponding bones in the forelimb.[12,13] Furthermore, alterations in *PITX1* expression underlie rapid evolutionary changes in pelvic morphology in vertebrate populations, including stickleback fish and manatee,[14,15] supporting an important role for *PITX1* in hindlimb development.

The opportunity to identify a gene responsible for idiopathic clubfoot arose from the identification of a five-generation North American family of European descent in which clubfoot segregates as an autosomal-dominant condition with reduced penetrance. In addition to bilateral clubfoot, the proband also manifests bilateral foot preaxial polydactyly, right-sided tibial hemimelia (Figures 1A and 1B), and a left-sided small preauricular skin tag (not shown). Five additional family members are affected with clubfoot; three of whom have increased severity on the right. One male has unilateral left clubfoot. Two individuals have bilateral oblique talus, manifesting as pes planus (flatfoot). These two patients had never been treated or evaluated, whereas all six individuals with clubfoot underwent either surgical treatment or serial casting by an orthopaedic surgeon who diagnosed their condition as clubfoot.

Three individuals have bilaterally hypoplastic patella resulting in patellar dislocations in late childhood. One individual had developmental hip dysplasia, but he was born in breech presentation, a known risk factor for this condition. He also has pes planus. Other than the proband, no other family members have polydactyly or tibial

[1]Department of Orthopedic Surgery, Washington University School of Medicine, St Louis, MO 63119, USA; [2]Department of Neurology, Washington University School of Medicine, St Louis, MO 63119, USA; [3]Department of Pediatrics, Division of Genomic Medicine, Washington University School of Medicine, St Louis, MO 63119, USA; [4]Department of Genetics, Division of Human Genetics, Washington University School of Medicine, St Louis, MO 63119, USA; [5]University of Texas Health Sciences Center, Houston, TX 77030, USA; [6]Seay Center for Musculoskeletal Research, Texas Scottish Rite Hospital for Children, Dallas, TX, USA; [7]St. Louis Shriners Hospital for Children, St. Louis, MO 63131, USA
*Correspondence: gurnettc@neuro.wustl.edu
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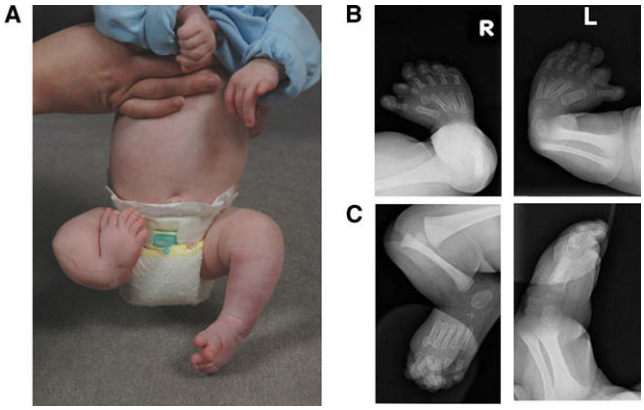

Figure 1. Complex Bilateral Congenital Abnormalities of the Lower Extremities of Proband of Family CF5171 Showing Greater Involvement on the Right Side

(A) Photograph of the proband demonstrating bilateral duplicated great toes and bilateral clubfoot. The clubfoot is more severe on the right.

(B) Radiographs of the digits and rays of both feet, showing bilateral duplication of the first toe, as well as partial duplication of the first metatarsal on the right foot.

(C) Radiographs of the lower limb showing absence of the tibia on the right only.

hemimelia. No upper extremity abnormalities or dysmorphic craniofacial features were noted. There are five clinically unaffected female carriers and no clinically unaffected males.

After informed consent and approval were obtained from the local human ethics review committees, a genome-wide linkage scan was performed on 13 family members with Affymetrix 10K mapping SNP genotypes. Linkage was performed assuming autosomal-dominant inheritance with 70% penetrance, a disease allele frequency of 0.01%, no phenocopies, and Affymetrix "Caucasian" allele frequencies. Parametric multipoint logarithm of the odds (LOD) score analysis performed with sets of 60–100 markers with GENEHUNTER[16] yielded a maximum LOD score of 3.31 with markers on chromosome 5q23.3-q31.2 (Figure 2). Recombinants delineated the critical genetic region to a 9 cM interval between SNP_A-1507970 and SNP_A-1517278, corresponding to a 12 Mb region (chr5:126,890,333-138,147,299) (Figure 3). The chromosome 5q23.3-q31.2 candidate region contains more than 80 genes.

Sequencing of *PITX1*, a bicoid-related homeodomain gene located within the candidate interval, revealed a single missense nucleotide change c.388G→A that results in the substitution of lysine for glutamic acid (E130K) in the highly conserved homeodomain (Figure 4A). This glutamic acid is at position 42 of the homeodomain within the DNA-binding third helix and is located near the Lys50 residue that imparts bicoid-type DNA-binding specificity.[17] The *PITX1* E130K mutation was present in all affected family members as well as in two tested obligate carriers but was absent in 500 white North American control subjects. On the basis of the NMR solution structure of the PITX homeodomain bound to DNA, the glutamic-acid side chain at position 42 (corresponding to the E130K mutation) extends into the major groove of DNA when bound[18] (Figure 4B). Glutamic acid at position 42 is conserved in all human PITX family members, across species, and in other nonbicoid homeodomain genes, including PAX7 (Figure 4C).

To characterize the effects of the *PITX1* E130K substitution on transcription-factor activity, we measured its ability to transactivate a luciferase reporter consisting of a thymidine kinase promoter preceded by four bicoid-binding sites (TAATCC).[19] COS7 cells were used for these experiments because they do not express PITX1 endogenously. The *PITX1* E130K mutation did not affect its expression or nuclear localization in transiently transfected COS7 cells (Figures 5A and 5B). Wild-type (WT) PITX1 transactivated a luciferase reporter gene ~7-fold, whereas the PITX1 E130K mutant activated the same reporter only ~4-fold (Figure 5C), demonstrating reduced transactivation activity.

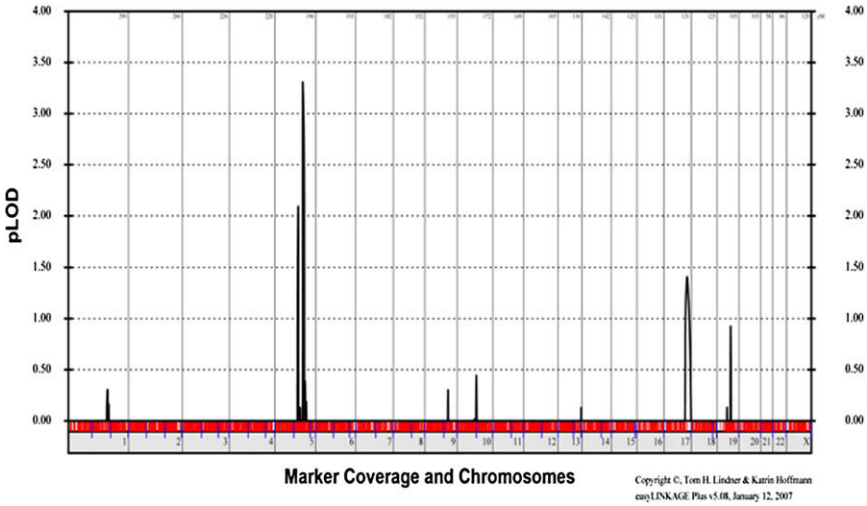

Figure 2. Linkage Analysis of Family 5171 with Multiple Lower-Limb Malformations

Genome-wide parametric multipoint linkage was performed with GENEHUNTER[16] with Affymetrix 10K array data from family 5171. Autosomal-dominant inheritance with 70% penetrance, a disease allele frequency of 0.01%, and no phenocopies were assumed for the analysis. Human chromosomes are concatenated from the p terminal (left) to the q terminal (right) on the x axis. The LOD score (y axis) is correlated to the physical location of human chromosome on the x axis and displayed with EasyLinkage software.[41,42]

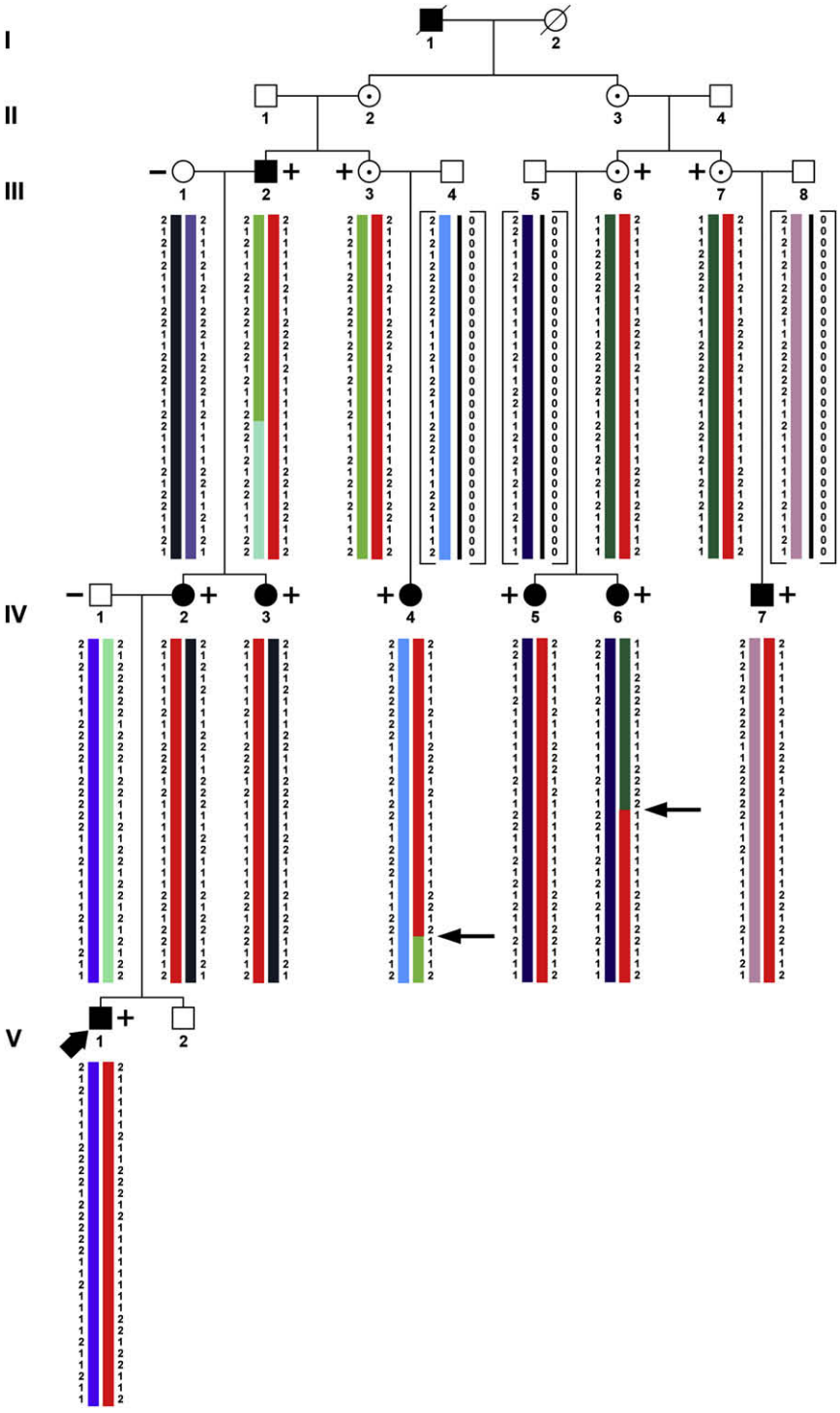

Figure 3. Haplotype Analysis Indicating Markers on Chromosome 5q23.3-q31.2 Common to All Affected Individuals

Haplotypes were created with GENEHUNTER and viewed on Haplopainter.[43] Presence or absence of the *PITX1* E130K mutation is noted with (+) or (−). The arrow indicates the proband.

Because bicoid-related homeodomain proteins form functional homodimers,[20] we examined whether the PITX1 E130K mutant might exert dominant-negative inhibition of WT PITX1 transactivation. Cotransfection of equal amounts of *PITX1* E130K plasmid DNA with WT *PITX1* reduced the ability of WT PITX1 to activate the luciferase reporter in COS7 cells (Figure 5C, lane 4). Negative effects of *PITX1* E130K mutant expression were also demonstrated on endogenous PITX1 in HeLa cells (data not shown). A dose-response curve demonstrated that the addition of increasing quantities of transfected WT *PITX1* DNA had no effect on luciferase activation, but increasing *PITX1* E130K DNA resulted in a dose-dependent decrease in luciferase activity (Figure 5D), supporting a dominant-negative effect of the PITX1 E130K mutant protein on transcriptional activation by *PITX1*.

Alterations of *PITX1* expression have been shown to underlie evolutionary adaptation of vertebrate hindlimb structures,[14,15] but this report is to our knowledge the first evidence for *PITX1* mutation in human disease. Exclusive involvement of the lower limb in individuals with the *PITX1* E130K mutation is consistent with the preferential expression of *PITX1* in the hindlimb[8,9] and its known role in hindlimb development.[11–13] Other known genetic causes of tibial hemimelia were also excluded in this family, including mutations in the long-distance sonic hedgehog enhancer[21,22] and duplications of the SHFM3 locus on chromosome 10q24-q25,[23] although both of these disorders are also associated with upper extremity involvement. Additional evidence that the *PITX1* E130K mutation is responsible for these lower-extremity birth defects is shown by the high degree of evolutionary conservation at the site of the mutation within the critically important DNA-binding homeodomain, as well as by the negative functional effects of this mutation on transcriptional activity.

Few transcriptional targets of PITX1 are known in the developing limb, with the exception of *TBX4*,[11] a transcription factor that is likewise preferentially expressed in the lower limb.[10] Several phenotypic similarities are present between patients with small patella syndrome (MIM 147891) caused by *TBX4* mutations[24] and patients described here with *PITX1* mutations, including patellar

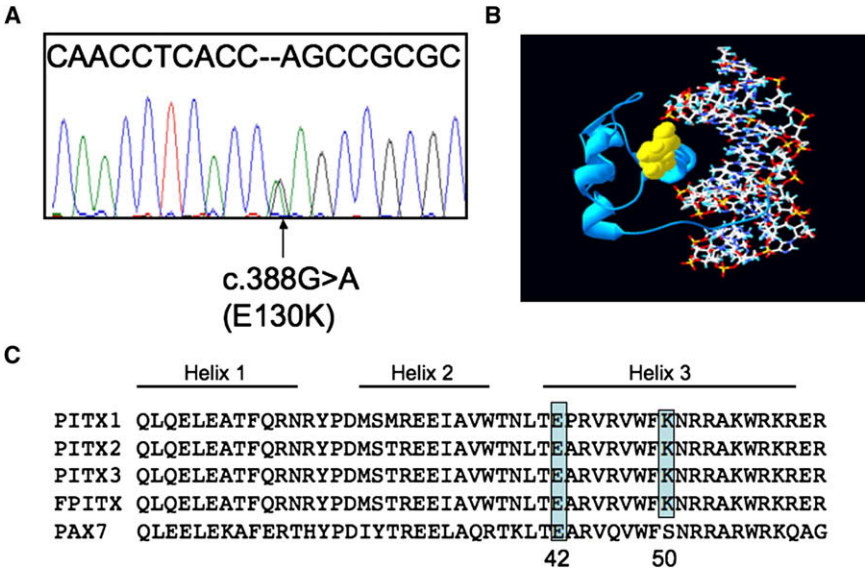

Figure 4. Identification of a *PITX1* Mutation in the Highly Conserved Homeodomain Segregating with Clubfoot in Family 5171

(A) Chromatogram showing PITX1 mutation c.388G→A resulting in missense mutation E130K.

(B) Ribbon diagram of the PITX2 homeodomain-DNA helix showing the position of the side chain (yellow) of the glutamic acid at position 42 corresponding to PITX1 E130K. The PITX1 E130K mutation lies within the critically important homeodomain third helix (shown to the left in blue, viewed end on) that interacts directly with the major groove of DNA (multicolored at right). We used the Swiss PDB DeepView program (see Web Resources) to visualize the corresponding structural location of the *PITX1* E130K mutation on the *PITX2* sequence bound to its consensus DNA site (TAATCC)[18] (accession code 1YZ8). Within the homeodomain shown, there is 97% amino acid identity between PITX1 and PITX2.

(C) Glutamic acid at homeodomain position 42 (corresponding to PITX1 E130K) is conserved in all human PITX family members, across species including Fugu (*Takifugu rubripes*), and in other nonbicoid homeodomain genes, including PAX7. Sequences were aligned with CLUSTALW (see Web Resources).

hypoplasia and pes planus. Asymmetric involvement has not been described in small patella syndrome. Characteristic foot abnormalities, including shortened fourth and fifth rays and wide spacing between the first and second toes, commonly seen with *TBX4* mutations,[25] appear to be uncommon in individuals with the PITX1 E130K mutation. Variable expressivity is common in both genetic disorders.

Mutations in all three known PITX bicoid-related homeobox-gene family members result in autosomal-dominant human congenital disorders. *PITX2* mutations were the first to be described in individuals with Rieger syndrome,[26] a disorder characterized by ocular anterior chamber anomalies, craniofacial dysmorphisms, dental hypoplasia, and umbilical stump abnormalities, whereas *PITX3* mutations result in autosomal-dominant cataracts and anterior segment mesenchymal dysgenesis.[27] Although many missense and nonsense *PITX2* mutations are presumed to cause Rieger syndrome through a loss of function,[26] a dominant-negative mutation was previously described (K88E) at homeodomain position 50,[28] which is located near the *PITX1* E130K mutation (homeodomain position 42) described here.

Asymmetric involvement is a hallmark of altered *PITX1* expression, as shown by the right-sided predominance of human lower-extremity malformations caused by the *PITX1* E130K mutation. Similarly, loss of *PITX1* expression in stickleback fish results in a greater reduction of pelvic structures on the right.[14] Manatee vestigial pelvic structures also show left-right directional asymmetry, suggesting that similar evolutionary mechanisms were responsible for the transition of this marine mammal from a four-legged terrestrial ancestor.[15] Loss of *Pitx1* expression in mice also causes greater shortening of femur length and loss of digits on the right foot as compared to the left.[12,13] This directional asymmetry has been ascribed to the unmasking of residual *PITX2* activity resulting from the loss of *PITX1* expression.[29] *PITX2* belongs to a small group of genes important for right-left body-axis patterning that results from its preferential expression in left lateral-plate mesoderm.[30–33]

Asymmetric lower-limb reduction malformations have frequently been described in familial tibial hemimelia, in which the right side is nearly always preferentially affected.[34,35] The consistent asymmetry noted in tibial hemimelia prompted Wiedemann and Opitz to propose a mechanism of "developmental resistance"[34] in which the left lower limb was protected from the effect of the mutation. Although vascular differences between left and right umbilical vessels have also been proposed as a possible mechanism leading to directional asymmetry,[36] molecular differences between limbs may account for most of this asymmetry.[37] Relative levels of *PITX1* and *PITX2* expression in the developing limb may be the first example of such a molecular signal that contributes to the overall propensity for right-sided malformations in an affected family or population. However, on an individual basis, other environmental or genetic effects may overcome this tendency, as demonstrated by the unilateral left-sided clubfoot seen in one individual with the *PITX1* E130K mutation. Occasional stickleback fish with left-sided pelvic-spine reduction and *PITX1* loss have also been described.[15,38]

Several clinical characteristics of individuals with the *PITX1 E130K* mutation suggest that *PITX1* and/or its pathways may be involved in the etiology of idiopathic

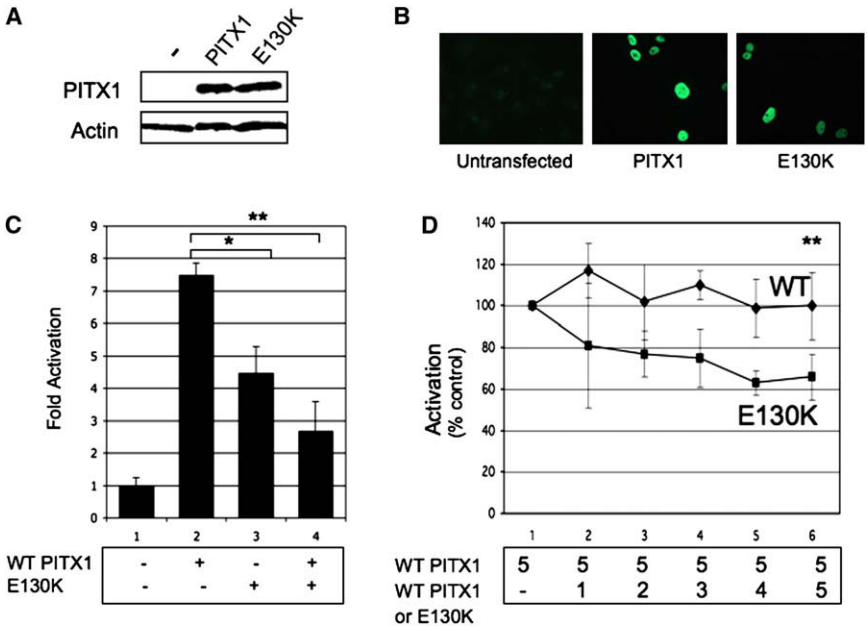

Figure 5. Reduced Transactivation Ability and Dominant-Negative Effects of *PITX1* E130K Mutation

(A) Western blot of equal amounts of COS7 cell extracts from a single transfection experiment probed with PITX1 antibody or actin antibody. COS7 cells were transfected with the TK-Bic reporter DNA alone (lane 1) or cotransfected with WT *PITX1* (lane 2), or *PITX1* E130K plasmids (lane 3).

(B) Immunofluorescence staining of transfected COS7 cells showing absent staining with PITX1 antibody in untransfected cells, and similar nuclear staining in cells transfected with either WT *PITX1* or *PITX1* E130K plasmid.

(C) Results of a luciferase reporter assay from COS7 cells transfected with TK-Bic reporter DNA alone or cotransfected with WT *PITX1*, *PITX1* E130K, or equal amounts of both WT *PITX1* and *PITX1* E130K mutant. There was reduced transactivation ability of *PITX1* E130K mutant compared to WT *PITX1* (*p = 0.034). Cotransfection of equal amount of PITX1 E130K with WT PITX1 also resulted in reduced activity compared to WT PITX1 alone (**p = 0.016). Activity is shown as mean fold activation compared to reporter alone ±SE from three independent experiments performed in triplicate.

(D) Dose-dependent decrease in luciferase reporter activation by PITX1 when cotransfected with varying amounts of *PITX1* E130K plasmid. COS7 cells were transfected with TK-Bic luciferase reporter, WT *PITX1* plasmid, and the indicated amount (ug) of either *PITX1* E130K mutant or WT *PITX1*. Activation was reduced when WT *PITX1* plasmid was cotransfected with *PITX1* E130K mutant compared to cotransfection with equal amount (5 ug) of WT *PITX1* (*p = 0.03). The data represent the three independent experiments with the mean fold activation ±SE.

clubfoot. First, the majority of the affected individuals in this family have isolated clubfoot with no other abnormalities. Second, incomplete penetrance was noted with the presence of five carrier females; such a finding is consistent with the lower incidence of idiopathic clubfoot in females.[3] Third, tibial hemimelia and clubfoot affect the right foot more frequently,[3, 35,39] suggesting the possibility that *PITX1* or its pathways may contribute to this directional asymmetry. However, we were unable to identify any additional *PITX1* coding mutations in 100 patients with clubfoot and eight with tibial hemimelia, including probands from three separate multigenerational families in which exclusively right-sided lower-limb malformations segregate. Failure to identify additional mutations in our study was not entirely unexpected, as studies in stickleback fish also failed to demonstrate *PITX1* mutations, despite strong linkage to the region and evidence of altered gene expression.[14] Many highly conserved noncoding regions potentially corresponding to limb-specific regulatory elements occur within the >300 kb gene desert surrounding *PITX1* and mutations in these regions may result in a similar phenotype. It is also possible that mutations in genes regulating PITX1 expression or other early expressed genes involved in establishing left-right asymmetry[40] may cause asymmetric lower-limb malformations.

Our results demonstrate a role for the bicoid-related homeobox gene PITX1 in a variety of human lower-limb malformations, including clubfoot, pes planus, tibial hemimelia, and patellar hypoplasia. The asymmetric hallmark of altered PITX1 expression seen in vertebrates as diverse as humans, mice, stickleback fish, and manatee supports the possibility that PITX1 or its pathways may be etiologically responsible for the increased incidence of right-sided tibial hemimelia and clubfoot. Furthermore, implication of a transcription-factor gene involved in early limb development suggests additional pathways for the future investigation of idiopathic clubfoot etiology in humans.

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Web Resources

The URLs for data presented herein are as follows:

Mulitple sequence Alignment by CLUSTALW, http://align.genome.jp

Online Mendelian Inheritance of Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim

Swiss PDB DeepView program, http://ca.expasy.org/spdbv

UCSC Genome Browser, http://genome.ucsc.edu/cgi-bin/hgGateway

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