

Deletions in *HOXD13* Segregate with an Identical, Novel Foot Malformation in Two Unrelated Families

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Summary

Synpolydactyly (SPD) is a dominantly inherited congenital limb malformation consisting of 3/4 syndactyly in the hands and 4/5 syndactyly in the feet, with digit duplication in the syndactylous web. The condition recently has been found to result from different-sized expansions of an amino-terminal polyalanine tract in *HOXD13*. We report a novel type of mutation in *HOXD13*, associated in some cases with features of classic SPD and in all cases with a novel foot phenotype. In two unrelated families, each with a different intragenic deletion in *HOXD13*, all mutation carriers have a rudimentary extra digit between the first and second metatarsals and often between the fourth and fifth metatarsals as well. This phenotype has not been reported in any mice with genetic modifications of the *HoxD* gene cluster. The two different deletions affect the first exon and the homeobox, respectively, in each case producing frameshifts followed by a long stretch of novel sequence and a premature stop codon. Although the affected genes may encode proteins that exert a dominant negative or novel effect, they are most likely to act as null alleles. Either possibility has interesting implications for the role of *HOXD13* in human autopod development.

Introduction

Synpolydactyly (SPD; MIM 186000) is a rare, dominantly inherited limb malformation that typically consists of 3/4 syndactyly in the hands and 4/5 syndactyly in the feet, with digit duplication in the syndactylous

web (Temtamy and McKusick 1978). Incomplete penetrance and variable expressivity, both between and within affected families, are common (Thomsen 1927; Cross et al. 1968; Sayli et al. 1995). One to four limbs can be involved, and the severity of involvement varies from partial skin syndactyly to almost complete digit duplication, extending proximally as far as the metacarpals/metatarsals, which may have a characteristic bifid appearance on radiological examination. Associated distal limb abnormalities include clinodactyly, camptodactyly, or brachydactyly of the fifth fingers and middle phalanx hypoplasia/aplasia, sometimes with syndactyly, of the second to fifth toes.

SPD is the first human malformation syndrome shown to be caused by mutations in a *HOX* gene, *HOXD13* (Muragaki et al. 1996). The mutation in question is specific and highly unusual. An imperfect trinucleotide repeat sequence in exon 1 of the gene, which in normal individuals encodes a 15-residue polyalanine tract, is expanded, resulting in a tract of 22–29 residues (Akarsu et al. 1996; Muragaki et al. 1996; Goodman et al. 1997). Studies of affected families with different-sized expansions show that both penetrance and severity of phenotype increase progressively with increasing expansion size (Goodman et al. 1997). This close correlation, together with the observation that the limb phenotype of mice lacking *Hoxd13* is strikingly different from that of humans with SPD (Dollé et al. 1993; Davis and Capecchi 1996), has led to the suggestion that expansions of the polyalanine tract may confer a progressive gain of function on the mutant protein. This hypothesis is supported by the finding that mice homozygous for a deletion encompassing *Hoxd11*, *Hoxd12*, and *Hoxd13* have central digital fusions and duplications resembling those of patients with SPD (Zákány and Duboule 1996), implying that the mutant *HOXD13* protein in SPD may be exerting a dominant negative effect over the remaining wild-type *HOXD13*, as well as over *HOXD11* and *HOXD12*.

To explore further the role of *HOXD13* in human limb development, we sought to identify patients with phenotypes similar to that of classic SPD in whom there

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was no evidence of a *HOXD13* polyalanine tract expansion. We now report our findings in two such families, each carrying a different intragenic deletion in *HOXD13* which segregates with distal limb abnormalities. Although some carriers of each deletion showed features of classic SPD, all deletion carriers from both families shared a novel foot malformation.

Patients and Methods

Patients

The two families that we report were ascertained after referral for genetic counseling to the Human Genetics Service, University of Florence (pedigree 1), and the Clinical Genetics Unit, Great Ormond Street Hospital, London (pedigree 2). In each case, the study was undertaken with the approval of the local ethical review board. Pedigree 1 (fig. 1A) was a six-generation Italian family. Digital abnormalities were initially identified in three family members: by clinical examination, for individual VI-1 (the proband); by means of an old family photograph, for individual III-5 (who was unavailable for clinical evaluation); and on the basis of family reports, for individual II-3 (who was deceased). Ten additional family members (indicated in fig. 1A) were subsequently examined clinically, and radiographs of both hands and both feet were obtained for five of these individuals (III-4, IV-2, IV-4, V-3, and V-4). Pedigree 2 (fig. 1B) was a five-generation Scottish family. Digital abnormalities were initially identified in seven family members: by clinical examination, for individuals III-2, III-4, IV-2 (the proband), IV-4, and V-1; and on the basis of family reports, for individuals II-5 and II-6 (both deceased). Three additional family members (indicated in fig. 1B) were subsequently examined clinically. Radiographs of both hands and both feet were obtained for seven family members (indicated in fig. 1B). In the case of individuals III-2, IV-2, and IV-4, who had undergone corrective surgery in childhood, these were postoperative films; for all three, however, the original extent of the malformation could be established from old medical records. For both pedigrees, information about the clinical status of those family members who were either deceased or unavailable for examination was sought from the family. Venous blood samples were obtained from consenting individuals, 13 from pedigree 1 and 11 from pedigree 2 (indicated in figs. 1A and 1B).

Methods

Genomic DNA for mutation analysis was extracted from whole venous blood samples (QIAamp Blood Kit, QIAGEN). To screen for expansions of the *HOXD13* polyalanine tract, we performed PCR using primers designed to amplify a 172-bp fragment of exon 1 of

HOXD13, which contains the imperfect trinucleotide repeat sequence encoding the tract. Primers and reaction conditions were as described elsewhere (Muragaki et al. 1996). Products were analyzed on a 4% agarose gel to detect the presence of an expanded allele. To search for mutations elsewhere in the gene, we amplified the entire *HOXD13* coding sequence by PCR in three segments, using the primers and reaction conditions described elsewhere (Goodman et al. 1997). Products either were subcloned into pCRScript (Stratagene) before being cycle sequenced (Applied Biosystems Prism Dye Terminator Kit) or were cycle sequenced directly.

After identification of the deletion in the proband from pedigree 1, mutation screening in other family members was performed by PCR using sense primer 5'-CA-GTGCCGCGGCTTTCTCT-3' and antisense primer 5'-GACATACGGCAGCTGTAGTAGC-3'. These yielded a 308-bp product starting at base 91 of the coding sequence in normal individuals and a second 294 bp product in the proband. (Numbering of bases in this report starts with the first residue of the initiator codon [GenBank accession numbers AF005219 and AF005220].) Amplification reactions contained 1 × *Pfu* buffer (Stratagene), 0.25 mM of each dNTP, 10% glycerol, 2% formamide, 1 μM of each primer, and 0.6 U of cloned *Pfu* polymerase (Stratagene) in a final volume of 25 μl. Initial denaturation was at 97.5°C for 2 min, followed by 30 cycles of denaturation at 97.5°C for 30 s, annealing at 61°C for 30 s, and extension at 72°C for 45 s. PCR products were analyzed on a 4% agarose gel to detect the presence of the deleted allele.

After identification of the deletion in the proband from pedigree 2, we performed mutation screening in other family members, using PCR and restriction site analysis. We amplified a 335-bp fragment containing exon 2 of *HOXD13*, using the primers and reaction conditions described elsewhere (Goodman et al. 1997). A *PstI* site occurs after base 135 of this fragment, which is destroyed by the deletion of the single G residue identified in the proband. *PstI* digestion reactions contained 5 U of *PstI* (Gibco BRL), 1 × REact 2 buffer (Gibco BRL) and 50–100 ng PCR product in a final volume of 15 μl. Digestions were carried out at 37°C for 2 h, and the products were analyzed on a 4% agarose gel.

Results

Mutation Analysis

In each family, initial screening of the proband by PCR showed that the *HOXD13* 15-residue polyalanine tract, which is expanded in all SPD patients studied to date, was normal in length.

In pedigree 1, sequence analysis of *HOXD13* in the proband revealed a 14-bp deletion in exon 1 (figs. 2A

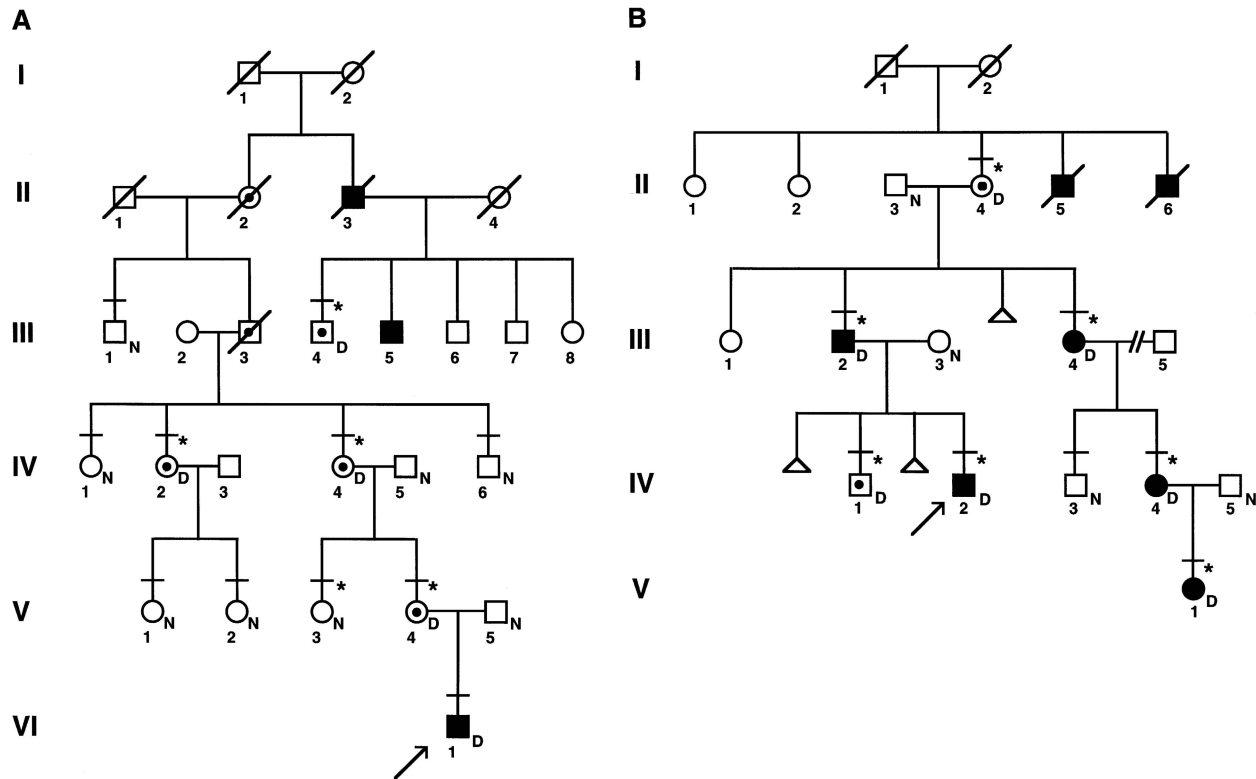


Figure 1 A, Pedigree 1. B, Pedigree 2. A horizontal line above a pedigree symbol indicates that the individual was examined clinically. An asterisk above a pedigree symbol indicates that radiographs of both hands and both feet were obtained. The letter “D” or “N” below a pedigree symbol indicates that DNA analysis was carried out, which demonstrated that the individual either did (D) or did not (N) carry the familial deletion. Blackened symbols represent individuals in whom a digital abnormality was identified on clinical examination, present on an old photograph (III-5 in pedigree 1), or reported by the family (II-3 in pedigree 1, II-5 and II-6 in pedigree 2). Unblackened symbols represent individuals who had no digital abnormality on clinical examination or according to family reports. Dotted symbols represent individuals found to be normal on clinical examination but mutation carriers on DNA analysis, or, in the case of II-2 and III-3 (both deceased) in pedigree 1, individuals who were unaffected according to the family but were obligate mutation carriers on pedigree analysis. In both families, pedigree analysis indicated that either I-1 or I-2 was a mutation carrier, although all four individuals (now deceased) were reported to have been unaffected. Because it is impossible to determine which of these individuals did in fact carry the mutation, all four symbols have been left unblackened.

and 2B), encompassing bases 323–336 of the coding sequence. This deletion begins 134 bases downstream of the end of the imperfect trinucleotide repeat sequence that encodes the 15-residue polyalanine tract. Interestingly, it affects a region of exon 1 (bases 313–330) that encodes a second polyalanine tract, in this case only six alanine residues long. PCR amplification of a 308-bp fragment of exon 1 containing the 14-bp deleted region yielded a second, 294-bp product in the proband, thus providing a simple means of screening other family members for the presence of the mutation. In this way, the deletion was also detected in four additional family members: III-4, IV-2, IV-4, and V-4 (results not shown). This deletion creates a frameshift leading to a premature stop codon at bases 681–683 of the coding sequence, which would be expected to result in a protein containing only the first 107 amino acids of the wild-type pro-

tein, followed by 115 amino acids with no counterpart in the wild-type protein and entirely lacking the homeodomain (fig. 3C).

In pedigree 2, sequence analysis of *HOXD13* in the proband revealed that a single G residue, the 77th residue of exon 2 (base 834 of coding sequence), was deleted (figs. 2C and 2D). This deletion destroys a *Pst*I site in a 335-bp fragment produced by PCR amplification of the exon. After digestion, the two expected 135-bp and 200-bp bands were detected in normal individuals, whereas a third, 335-bp band, representing the uncut PCR fragment, was also detected in the proband, confirming that he was heterozygous for the deletion. Again, this provided a convenient means of screening other family members for the presence of the deletion, allowing us to identify it in six additional family members: II-4, III-2, III-4, IV-1, IV-4, and V-1 (results not shown). This

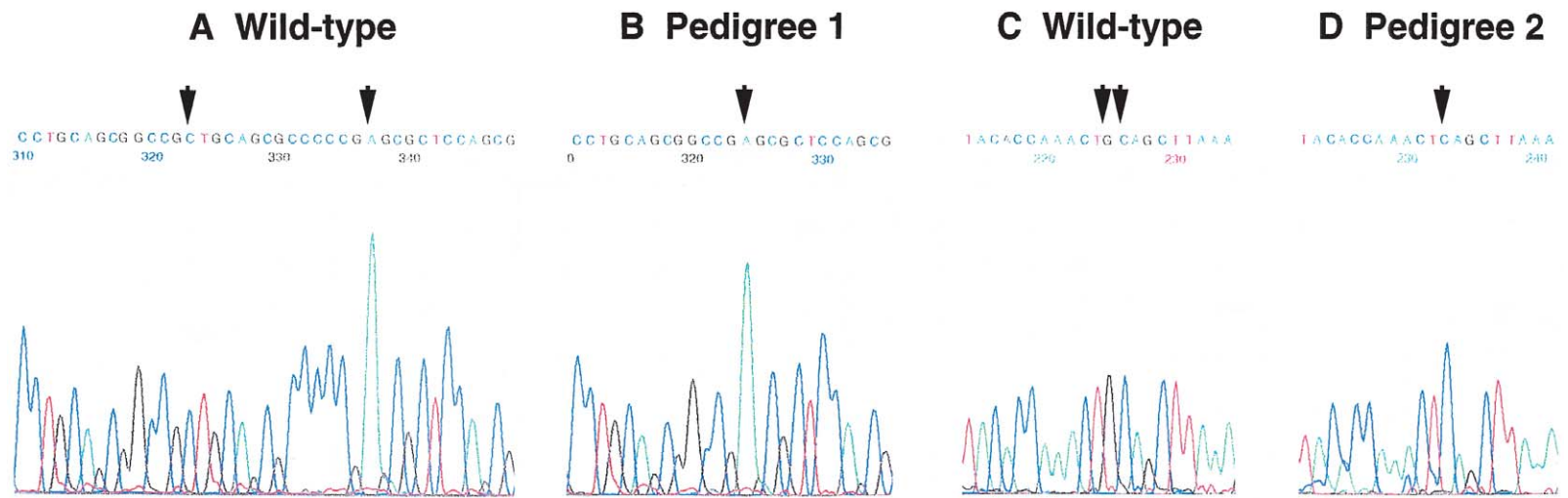


Figure 2 Sequence analysis of the two deletions. *A*, Bases 310–348 of the wild-type coding sequence. The 14 bases deleted in pedigree 1 (bases 323–336) lie between the two arrowheads. *B*, The same sequence in the proband from pedigree 1, showing the site of the deletion (arrowhead). *C*, Bases 823–843 of the wild-type coding sequence. The single G residue deleted in pedigree 2 (base 834) lies between the two arrowheads. *D*, The same sequence in the proband from pedigree 2, showing the site of the deletion (arrowhead).

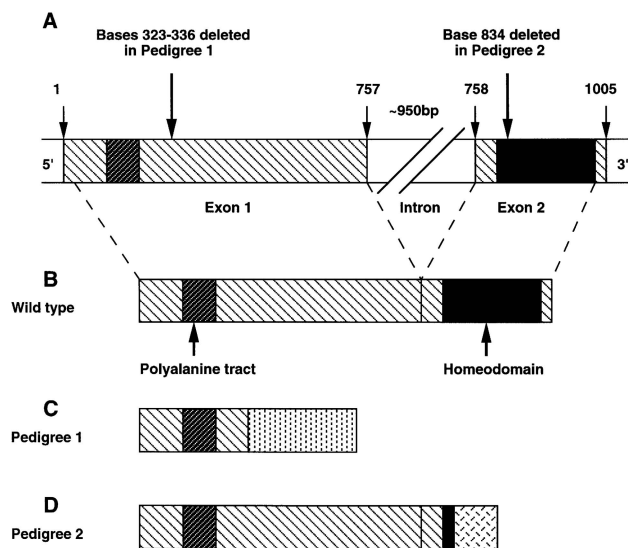


Figure 3 A, The genomic structure of *HOXD13*, showing the sites of the two deletions. B, The wild-type *HOXD13* protein. C, The predicted mutant protein in pedigree 1. The first 107 wild-type amino acids are followed by 115 amino acids with no counterpart in the wild-type protein and by a premature stop codon. D, The predicted mutant protein in pedigree 2. The first 278 wild-type amino acids, including the first 11 amino acids of the homeodomain, are followed by 33 amino acids with no counterpart in the wild-type protein and by a premature stop codon.

deletion also creates a frameshift, leading to a premature stop codon at bases 935-937 of the coding sequence. The resulting protein would be expected to contain only the first 278 amino acids of the wild-type protein, followed by 33 amino acids with no counterpart in the wild-type protein, and would lack the last 49 amino acids of the 60-amino acid homeodomain, including the entire recognition helix (fig. 3D).

To exclude the possibility that polymorphisms or other mutations in *HOXD13* might cosegregate with the observed deletions and thus influence the phenotype, the entire mutant allele from one affected individual from each pedigree (IV-2 from pedigree 1 and III-2 from pedigree 2) was sequenced and found to be normal, apart from the deletions already identified (results not shown).

Phenotype Analysis

The distal limb phenotype in mutation carriers from both families is summarized in table 1. The most striking finding was a distinctive set of abnormalities present bilaterally in the feet of all 11 proven deletion carriers who were available for radiological examination (III-4, IV-2, IV-4, and V-4 from pedigree 1; and II-4, III-2, III-4, IV-1, IV-2, IV-4, and V-1 from pedigree 2). Partial duplication of the bases of the second metatarsals in the first web spaces was seen in every case, often accom-

panied by a similar duplication of the fourth metatarsals in the fourth web spaces. In addition, the phalanges of the halluces were abnormally broad and the first metatarsals were often short (figs. 4A and 4B). These features constitute a unique dominantly inherited foot phenotype that has not been reported previously. In particular, this phenotype does not occur in patients with classical SPD caused by *HOXD13* polyalanine tract expansions and extends as far anteriorly as the first digits, which are not normally affected in SPD. In addition, the middle phalanges in the second to fifth toes were often hypoplastic or fused to the distal phalanges, as sometimes occurs in classical SPD. This novel foot phenotype is identical in mutation carriers from the two different pedigrees and appears to be fully penetrant.

The 3/4 finger and 4/5 toe synpolydactyly characteristic of classical SPD was also noted in some mutation carriers from both families. In pedigree 1, individuals II-3, III-5, and VI-1 all had bilateral 3/4 synpolydactyly in the hands (fig. 4C), but only individual VI-1 had unilateral 4/5 foot synpolydactyly (fig. 4D). (The foot phenotype in individuals II-3 and III-5 could not be ascertained.) In pedigree 2, individuals III-2 and IV-4 had bilateral 3/4 synpolydactyly in the hands (fig. 4E); individuals II-5, II-6, IV-2, and IV-4 had bilateral 4/5 synpolydactyly in the feet; and individuals III-2, III-4, and V-1 had unilateral 4/5 synpolydactyly in the feet. The number of mutation carriers studied is too small to allow

Table 1
Distal Limb Phenotype in Mutation Carriers

Subject	Novel Foot Phenotype	3/4 SPD in Hands	4/5 SPD in Feet
Pedigree 1:			
I-1 or I-2	?	No	No
II-2	?	No	No
II-3	?	Bilateral	?
III-3	?	No	No
III-4	Yes	No	No
III-5	?	Bilateral	?
IV-2	Yes	No	No
IV-4	Yes	No	No
V-4	Yes	No	No
VI-1	?	Bilateral	Unilateral
Pedigree 2:			
I-2 or I-2	?	No	No
II-4	Yes	No	No
II-5	?	No	Bilateral
II-6	?	No	Bilateral
III-2	Yes	Bilateral	Unilateral
III-4	Yes	No	Unilateral
IV-1	Yes	No	No
IV-2	Yes	No	Bilateral
IV-4	Yes	Bilateral	Bilateral
V-1	Yes	No	Unilateral

NOTE.—A question mark (?) indicates that information is unavailable.

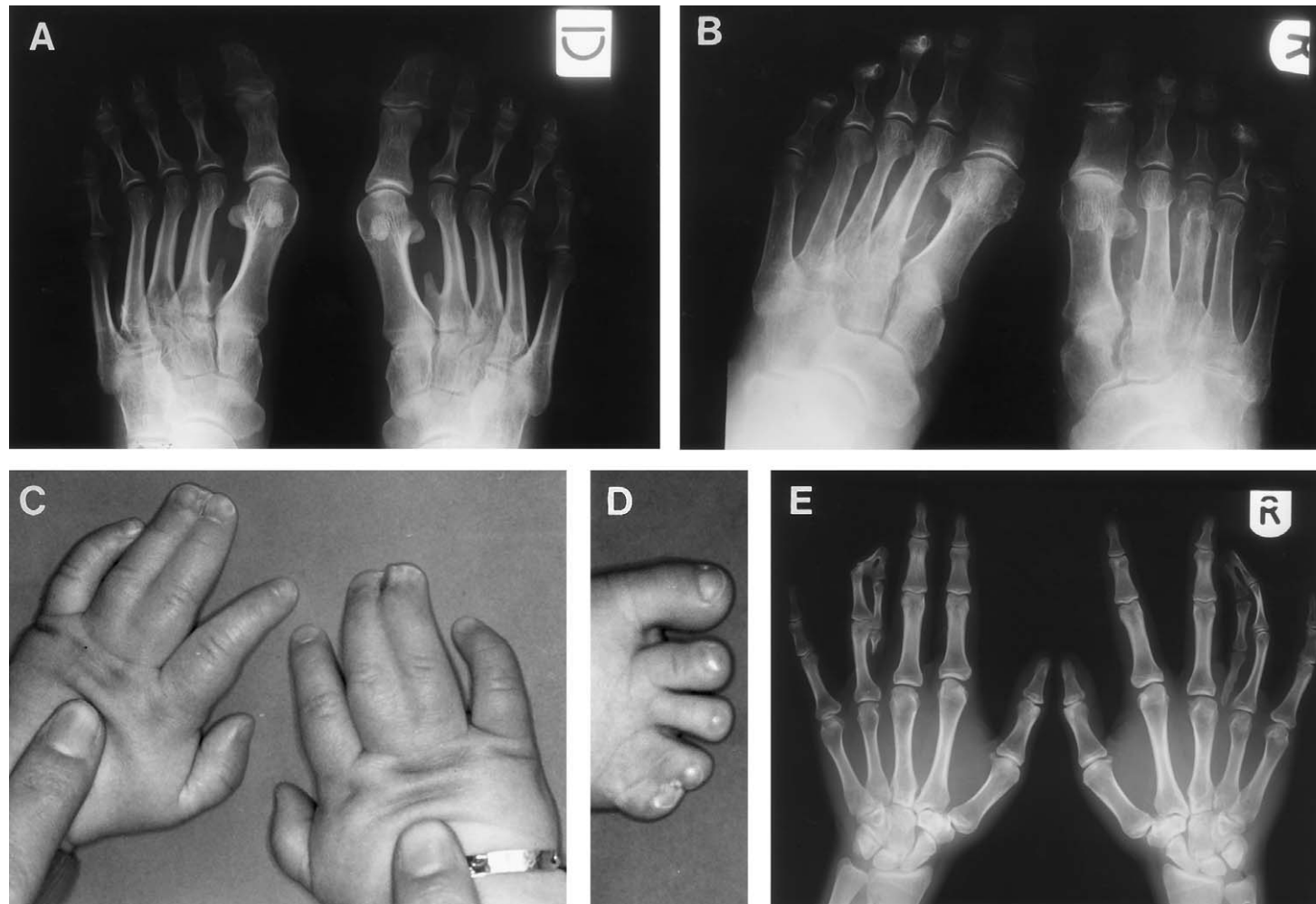


Figure 4 The phenotype in deletion carriers from the two pedigrees. *A* and *B*, The novel foot phenotype. *A*, Radiograph of both feet of V-4 from pedigree 1, age 33 years, showing partial duplication of the proximal second metatarsals in the first web spaces and partial duplication of the proximal fourth left metatarsal in the fourth web space. The distal phalanges of the halluces are broad, and there is symphalangism of the middle and distal phalanges of the second and fifth toes. *B*, Radiograph of both feet of II-4 from pedigree 2, age 72 years, showing bilateral partial duplications of the proximal second and fourth metatarsals in the first and fourth web spaces, respectively. The distal and proximal phalanges of the halluces are broad, and there is hypoplasia of the middle and distal phalanges of the second to fifth toes. *C*, *D*, and *E*, Features of classical SPD. Photographs of the hands (*C*) and the right foot (*D*) of the proband (VI-1) from pedigree 1, age 1 year, showing bilateral 3/4 syndactyly in the hands and unilateral 4/5 foot syndactyly. *E*, Radiograph of the hands of IV-4 from pedigree 2, age 25 years, after surgery in childhood to release bilateral 3/4 syndactyly, showing attempted duplication of the proximal, middle, and distal phalanges of both ring fingers, with soft-tissue syndactyly and bony syndactyly of the distal phalanges.

firm conclusions to be drawn about levels of penetrance. Nevertheless, the proportion of mutation carriers with 3/4 synpolydactyly in the hands appears to be similar in the two families (30% in pedigree 1 and 20% in pedigree 2), whereas the proportion of mutation carriers with 4/5 synpolydactyly in the feet is higher in pedigree 2 (70%) than in pedigree 1 (10%–30%). These data also suggest the intriguing possibility that 3/4 synpolydactyly may be less common in the hands of carriers of these deletions than in the hands of carriers of polyalanine tract expansions. Even in the case of seven-residue expansions (the smallest expansion known to produce a clinical phenotype and the expansion with the lowest penetrance), 58% of carriers have 3/4 synpolydactyly in the hands (28% have 4/5 synpolydactyly in the feet) (Goodman et al. 1997).

No mutation carrier in either family had any form of lower-urogenital tract malformation, although hypospadias has been noted in affected males from one SPD family with a 14-residue polyalanine tract expansion (Goodman et al. 1997).

Discussion

The two deletions in *HOXD13* reported here are associated with a novel foot phenotype that is characterized by preaxial as well as postaxial polydactyly and extends as far as digit 1, the extreme anterior border of *HOXD13* function during specification of the autopod (Nelson et al. 1996). Although mild, this phenotype appears to be fully penetrant. The more severe features of classical SPD, 3/4 synpolydactyly in the hands and 4/5 synpolydactyly in the feet, also occur, but at a reduced penetrance. Since the novel foot phenotype is never found in patients with classical SPD caused by polyalanine tract expansions, it represents a hitherto undescribed extension of the phenotypic spectrum seen in SPD, presumably resulting from a different perturbation of *HOXD* cluster function during autopod development. Moreover, the two deletions must act by the same or a very similar mechanism, since they produce virtually indistinguishable phenotypes in carriers from the two different families. In principle, the two mutations could exert their effects by (1) perturbing the ratio of normal transcripts from the mutant allele; (2) directing the synthesis of abnormal proteins, which then exert a dominant negative or novel effect; or (3) preventing the production of any stable protein, thus leading to functional haploinsufficiency. We consider these three alternatives in order.

First, a protein with a functional homeodomain could theoretically still be produced from both mutant alleles. There are several potential alternative initiator codons in exon 1 of the gene, and a shorter form of *Hoxd13* starting at codon 98 of the longer form has been reported

in developing chick limbs (Goff and Tabin 1997). This smaller isoform, if it occurs in humans, could still be produced from alleles carrying the exon 1 deletion, but this would not be possible in the case of the exon 2 deletion, which directly disrupts the homeodomain. Second, alternative splice forms of *HOXD13* may exist, as has been demonstrated for several other mammalian Hox proteins (Benson et al. 1995; Chariot et al. 1995; Hong et al. 1995; Fujimoto et al. 1998). In the case of the exon 1 deletion, a hitherto unidentified 5' exon could be spliced to exon 2, whereas in the case of the exon 2 deletion, exon 1 could be spliced to the second, homeobox-encoding exon of the adjacent *HOXD12* gene, in each case permitting synthesis of proteins with functional homeodomains and perturbing the resulting ratio of *HOXD13* isoforms. As yet, however, there is no evidence that more than one initiator codon or splicing pattern is used in *HOXD13*. Moreover, both choice of initiation site and splicing would be affected differently by the two mutations, and this is difficult to reconcile with the extremely similar phenotypes that they produce.

The second alternative is that an abnormal protein is expressed from both mutant alleles. Homeodomain proteins in which the homeodomain has artificially been partially deleted occasionally retain their capacity for protein/protein interactions when expressed at high levels (Copeland et al. 1996; Hyduk and Percival-Smith 1996), and transcripts lacking the homeobox have been reported for several mammalian *Hox* genes (Chariot et al. 1995; Fujimoto et al. 1998). The two truncated mutant proteins predicted here might thus exert a functional effect through their remaining amino-terminal domains. In both cases, the 15-residue polyalanine tract, expansions of which cause classical SPD, would remain intact. Given the extreme similarity of the phenotypes produced, however, such a functional effect would have to be mediated by only the first 107 wild-type residues (the region shared by the two mutant proteins), and these 107 residues would have to adopt the same fold when followed either by 115 novel residues (pedigree 1) or by 171 wild-type residues and 33 different novel residues (pedigree 2). If this is indeed the case, these initial 107 residues must play a crucial functional role in human autopod formation.

The final alternative is that no functional protein is made from either mutant allele. In addition to truncating the normal protein, both deletions also produce a long carboxy-terminal tract of nonsense sequence, ending with a premature stop codon. Most frameshift mutations of this kind have been found in practice to act as null alleles (for examples, see Hanson et al. 1993; Tassabehji et al. 1995; Willing et al. 1996; Edery et al. 1997), in part because transcription from the mutant allele is generally markedly reduced as a result of nonsense-mediated mRNA decay (Belgrader et al. 1994; Maquat 1995; Ja-

cobson and Peltz 1996). The two mutant proteins predicted here are thus unlikely to be synthesized at appreciable levels. Moreover, such tracts of carboxy-terminal nonsense sequence generally do not adopt a stable fold, resulting in rapid degradation of the entire protein (Kalderon 1996). A loss-of-function interpretation is also easier to reconcile with the observation that the two different mutant proteins appear to exert the same functional effect. We therefore favor the alternative that both mutations act as null alleles, although the other alternatives cannot at this stage be ruled out.

If this interpretation is correct, the phenotype described here represents the first report of functional haploinsufficiency for a *HOX* gene in humans (unless the nonsense mutation in *HOXA13* recently found in one family with hand-foot-genital syndrome [Mortlock and Innis 1997] also proves to inactivate the resulting protein). At first sight, however, it is inconsistent with the reported phenotypes of mice in which the *HoxD* gene cluster has been genetically modified.

Knockout mice lacking *Hoxd13* have been described by two groups (Dollé et al. 1993; Davis and Capecchi 1996). Only 36% of heterozygotes are affected, and those that are have minor carpal defects and an additional bony element postaxial to the fifth digit in the forelimb only. (Homozygotes show shortening of the second phalanges and the metapalanges, especially in digits 2 and 5, as well as a rudimentary extra digit postaxial to digit 5 in some cases.) If the patients described here are indeed haploinsufficient for *HOXD13*, then the strikingly different phenotype of these knockout mice might reflect differences in the role of *HOXD13* in murine and human autopod development. Because this process is highly conserved, however, this seems unlikely. More plausibly, replacement of the mouse gene's homeobox by the neomycin-resistance cassette might not produce a simple null *Hoxd13* allele, either because the intact first exon still encodes a functional product (Copeland et al. 1996; Hyduk and Percival-Smith 1996) or because the cassette itself interferes with the regulation of other 5' *Hoxd* genes (Ramírez-Solis et al. 1993; Rijli et al. 1994; Fiering et al. 1995; Olson et al. 1996; Pham et al. 1996).

Mice heterozygous for a deletion encompassing *Hoxd11*, *Hoxd12*, and *Hoxd13* merely have shortening of digits 2 and 5 (Zákány and Duboule 1996). (Homozygotes have phenotypes similar to human SPD heterozygotes and homozygotes, including an extra central digit fused to digit 3 or digit 4.) Although the different, more severe phenotype of the patients described here might again reflect differences between murine and human autopod development, it is also possible that it is due to the untrammelled action of *HOXD11* and *HOXD12*, whose effects are thought to be controlled

by *HOXD13* during normal autopod development (Zákány and Duboule 1996; Rijli and Chambon 1997).

Detailed *in vivo* studies of the functional effects of the two deletions reported here will be necessary to resolve which of the alternative explanations for the observed phenotype is correct. Whether or not the mutations indeed lead to functional haploinsufficiency, however, the resulting insights into the function of *HOXD13* will be certain to advance our understanding of human limb development.

Acknowledgments

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, <http://www.ncbi.nlm.nih.gov/Web/Genbank> (for the coding sequence of *HOXD13* [accession numbers AF005219 and AF005220])

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim> (for SPD [MIM 186000])

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