ONLINE MUTATION REPORT

A novel duplication in the *HOXA13* gene in a family with atypical hand-foot-genital syndrome

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Hypospadias, when the urethral opening is located on the ventral side of the penis, is one of the most common congenital malformations with an incidence of 3 per 1000 males.[1] Hypospadias is considered a complex trait caused by several genetic and environmental factors; low birth weight, for example, is associated with an increased risk for hypospadias.[2–5] Most cases of hypospadias are sporadic but about 10% of the boys have a relative with the malformation.[4 6–9] There are families with an autosomal dominant inheritance pattern of hypospadias.[4 10] Hypospadias is also a manifestation in some rare single gene traits affecting sex differentiation, for example, the X linked partial androgen insensitivity syndrome and the recessive 5-alpha-reductase deficiency.[11–13] However, these syndromes are characterised by severe hypospadias in association with other genital malformations such as cryptorchidism, bifid scrotum, and penoscrotal transposition.

Hand-foot-genital syndrome (HFGS) is an autosomal dominant syndrome that may include hypospadias.[14 15] HFGS is characterised by skeletal anomalies and urogenital malformations. The skeletal manifestations affect the distal limbs and include short, proximally placed thumbs with hypoplastic thenar eminences, ulnar deviation of the second finger, clinodactyly of the fifth finger, short, medially deviated halluces, brachydactyly of the second to fifth toes, and shortening of the carpals and tarsals. Typical urogenital abnormalities in females are bicornuate uterus, vaginal septum, and ectopic localisation of ureteric and urethral orifices. Vesicoureteral reflux and ureteropelvic obstruction has been observed in females as well as in males.[16] The syndrome was initially called hand-foot-uterus syndrome by Stern *et al*,[17] but the observation of hypospadias in some affected males prompted the change of nomenclature.[18 19]

The most specific finding in HFGS is the typical radiographic pattern described by Poznanski *et al*.[19] In the hands, this includes a shortened metacarpal, a pointed distal phalanx, and pseudoepiphysis of the metacarpal bones in the thumb. In the feet, metatarsal 1 and proximal phalanx 1 are short and the big toe deviates medially or, less commonly, laterally. Incomplete ossification and fusion of tarsal bones are also seen. Overall, the skeletal manifestations are invariable and highly penetrant, whereas the urogenital abnormalities show reduced penetrance and variable expression.

Eight families and four sporadic cases with characteristic features of HFGS have been described so far.[16–18 20–28] The phenotype varies both within and between these families. In 1997, it was reported that HFGS is caused by mutations in the *HOXA13* gene.[29]

The following mutations in *HOXA13* have been found in seven HFGS families: four different nonsense mutations (W369X, S136X, Q196X, and Q365X), one missense mutation (N372H), and two in frame insertions of 24 and 18 bp respectively.[20 23 26 29] In addition, an interstitial deletion removing the entire *HOXA* cluster was found in the family reported by Devriendt *et al*.[22]

The insertions are both located in the last of three polyalanine tracts in the first exon and result in additional polyalanines. Similar polyalanine tract expansions have been described in *HOXD13* in several families with synpolydactyly.[30–32] The insertions in *HOXA13* and *HOXD13* consist of cryptic expansions (GCA, GCC, GCG, GCT) rather than trinucleotide repeats, are stable through generations, and are believed to have originated through unequal crossing over.[33]

Three additional families with atypical features of HFGS have been described.[34–36] However, there are divergences from the classical description making the diagnosis less probable, as pointed out by Utsch *et al*.[26] Nevertheless, in the family reported as Guttmacher syndrome also including polydactyly, both a missense mutation in the homeobox region of the *HOXA13* gene and a dinucleotide deletion in the promoter was found.[37] In the family with Müllerian duct fusion defects and ear malformations reported by Goodman *et al*,[23] no mutation in the *HOXA13* gene was found. The genetic basis for the syndrome described by Longmuir *et al*[36] with mainly distal skeletal malformations has not been addressed.

The mutations in *HOXA13* and *HOXD13*, together with a mutation in *HOXA11* resulting in amegakaryocytic thrombocytopenia and radioulnar synostosis, are the only mutations in *HOX* genes described in man to date.[38] Characteristically, these abnormalities are discrete and may easily escape medical attention.

We report the identification of a novel mutation in the *HOXA13* gene in a six generation family originally ascertained

Key points

- Hypospadias, when the meatus is located on the ventral side of the penis, is a common malformation with genetic and environmental factors involved in the pathogenesis. We describe a large family with autosomal dominant inheritance of hypospadias, clinodactyly, and subtle foot malformations.
- A genome wide linkage analysis showed evidence for linkage to the short arm of chromosome 7. Sequence analysis of the *HOXA13* gene showed a heterozygous 18 bp duplication in the second polyalanine tract, resulting in six additional alanines.
- Mutations in the *HOXA13* gene have been shown to cause hand-foot-genital syndrome (HFGS). HFGS is a rare autosomal dominant trait affecting the distal limbs and genitourinary tract previously described in 12 families. This family is the largest reported so far with 27 affected subjects in six generations and displays a variant of HFGS with less severe skeletal abnormalities but higher penetrance of hypospadias.
- This finding illustrates the minor consequences of *HOX* gene mutations in humans and serves as an example of dominant inheritance of hypospadias.

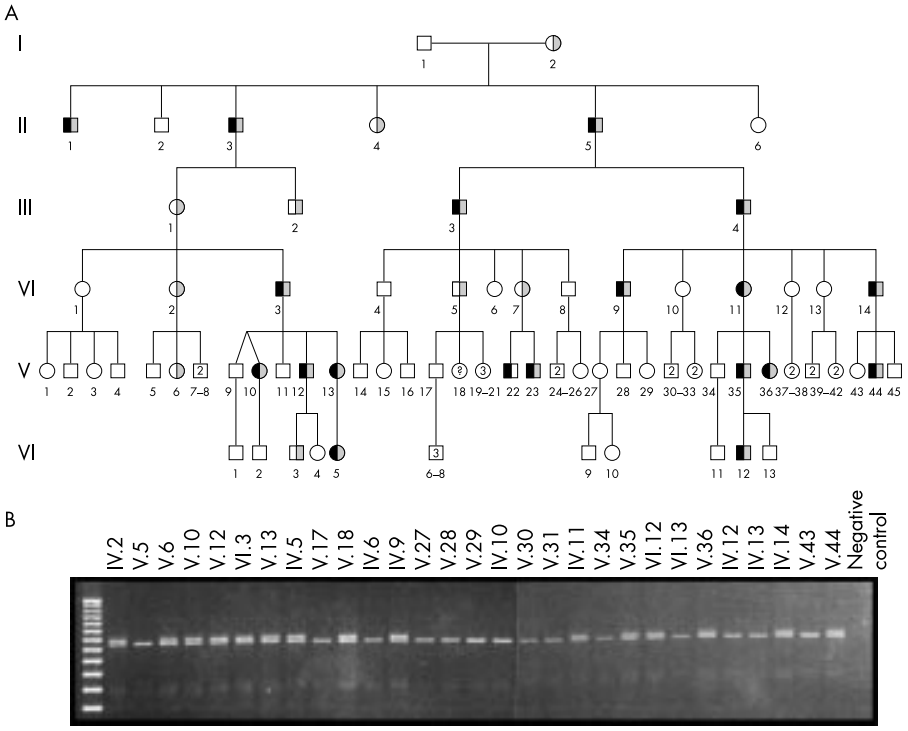

Figure 1 (A) Pedigree with phenotypic features as indicated: black shading of the left half: hypospadias in males, urogenital abnormalities in females; stippled right half: hand and/or foot abnormalities. (B) The PCR reaction results in a product size of 248 bp for the normal allele and 266 bp for the mutated allele.

because of the accumulation of hypospadias, which turned out to be an atypical variant of HFGS.

MATERIALS AND METHODS

Patients

The family with autosomal dominant inheritance of hypospadias was initially ascertained through the Department of Paediatric Surgery at Astrid Lindgren Children Hospital, Karolinska Hospital as part of an effort to map genes for hypospadias. Clinodactyly and mild foot malformations were present in both sexes (fig 1A). Phenotype information was acquired as described in table 1. We collected peripheral venous blood from available family member and isolated genomic DNA according to a standard protocol. The Ethics Committee at the Karolinska Hospital approved the study.

Linkage analysis

A genome wide linkage analysis was performed using 360 microsatellite markers with an average distance of 9.5 cM according to standard procedures.[39] An extended genotyping was subsequently carried out on chromosome 7 with the markers D7S2514, D7S641, D7S2464, D7S664, D7S2557, D7S2508, D7S507, D7S503, D7S488, D7S2551, D7S493, and D7S673. PCR conditions are available on request. PCR

Table 1 Phenotype data of affected family members

Patient	Sex	Skeletal abnormalities	Urogenital manifestations	Phenotype source
III.3	Male	Clinodactyly, short thumbs, small feet	Glandular hypospadias, micropenis	IV.4, IV.6, IV.11
III.4	Male	Clinodactyly, short thumbs, small feet	Glandular hypospadias	IV.4, IV.6, IV.12
IV.2*	Female	Clinodactyly		Interview with spouse
IV.3	Male	Small feet "without heels"	Chordee	Telephone interview
IV.5*	Male	Clinodactyly, hallux varus		Interview with relative
IV.7	Female	Clinodactyly, hallux varus		Interview with relative
IV.9*	Male	Clinodactyly	Glandular hypospadias	Telephone interview
IV.11*	Female	Clinodactyly, large gap between 1st and 2nd toe	Incontinence	Medical records
IV.14*	Male	Clinodactyly, small feet	Penile hypospadias, chordee	Interview with spouse
V.6*	Female	Clinodactyly		Telephone interview
V.10*	Female	Clinodactyly, large gap between 1st and 2nd toe, short 2nd toe	Incontinence	Telephone interview
V.12*	Male	Small feet	Glandular hypospadias	Telephone interview
V.13*	Female	Small feet	Recurrent urinary tract infections	Telephone interview
V.22	Male		Glandular hypospadias	Interview with relative
V.23	Male	Clinodactyly, hallux varus, short 2nd toe	Glandular hypospadias	Interview with relative
V.35*	Male	Clinodactyly, large gap between 1st and 2nd toe, short 2nd toe	Penoscrotal hypospadias, chordee	Examination by authors
V.36*	Female	Clinodactyly, large gap between 1st and 2nd toe, short 2nd toe	Incontinence	Examination by authors
V.44*	Male	Clinodactyly	Penile hypospadias	Interview with parent
VI.3*	Male	Clinodactyly, small feet, large gap between 1st and 2nd toe		Interview with grandparent
VI.5	Female	Clinodactyly, large gap between 1st and 2nd toe, short 2nd toe	Recurrent urinary tract infections	Medical records
VI.12*	Male	Clinodactyly	Glandular hypospadias	Examination by authors

*DNA analysed from these subjects.

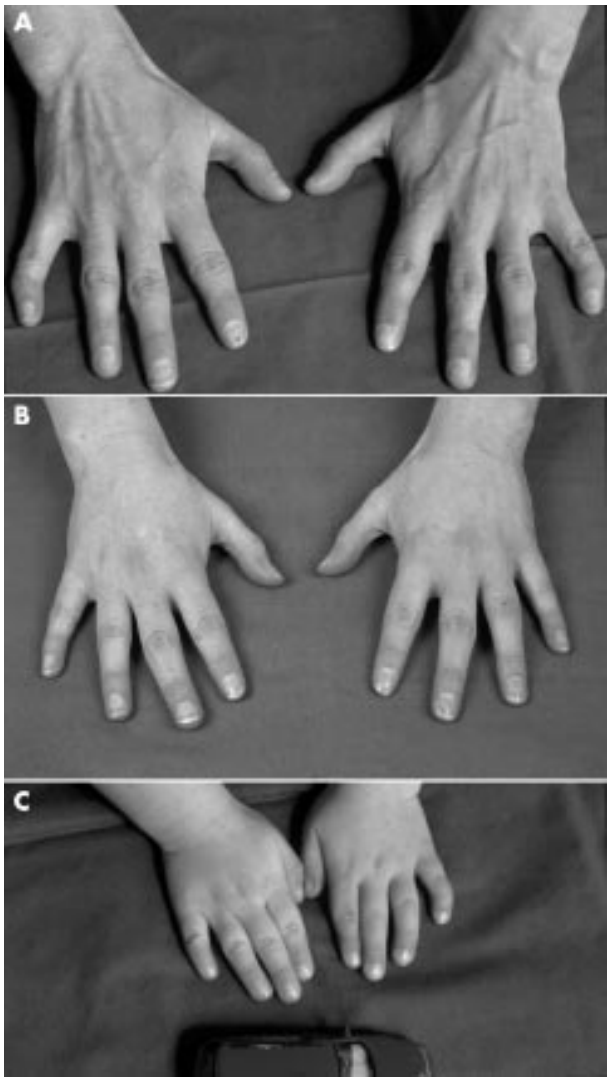

Figure 2 Hands of V.35, V.36, and VI.12, respectively. The malformation consists of a radial deviation of the distal phalanx of the little finger with variable expressivity.

products were separated by electrophoresis on an ABI377 (Applied Biosystems) and genotypes were scored in the GENOTYPER software. All genotypes were checked manually. Two point linkage analysis was performed using MLINK in the FASTLINK package through the HGMP web site (http://www.hgmp.mrc.ac.uk), assuming an autosomal dominant model with full penetrance and the gene frequency of 0.001.

Sequence analysis of the *HOXA13* gene

We used primers described by Kosaki *et al*,[40] with the exception that primer HOXA13-A67 was shortened with two nucleotides in the 3′ end to eliminate the large difference in annealing temperature between primers HOXA13-A67 and HOXA13-A68. Exon 2 of the *HOXA13* gene was amplified using primers HOXA13-ex2fwd (5′-CAGATCGAGCTGTCGCCTA-3′) and HOXA13-ex2rev (5′-TATCTGGGCAAAGCAACGA-3′). PCR reactions were performed in 10 mmol/l Tris-HCl pH 8.3, 50 mmol/l KCl, 2.0 mmol/l $MgCl_2$, 1.25 U Ampli*Taq* Gold (Applied Biosystems), 100 µmol/l dNTP (GibcoBRL), 0.1 µmol/l of each primer, and approximately 200 ng of genomic DNA in a total volume of 25 µl. We used the PCRx Enhancer System with 1X Enhancer solution (Invitrogen) when amplifying primers HOXA13-A65 and HOXA13-A66. PCR reactions were cycled in a 10 minute 96°C heat shock step followed by 35 cycles of a 30 second 96°C denaturation step, a 30 second 55-64°C annealing step, depending on the fragment to be amplified, and a two minute 72°C extension step.

DNA sequence analysis was performed on both strands of amplified and purified PCR products using the ABI PRISM BigDye Terminator Cycle Sequencing kit 2.0 (Applied Biosystems). The sequencing reactions were carried out according to the manufacturer's recommendations and analysed on an ABI310 DNA sequencer.

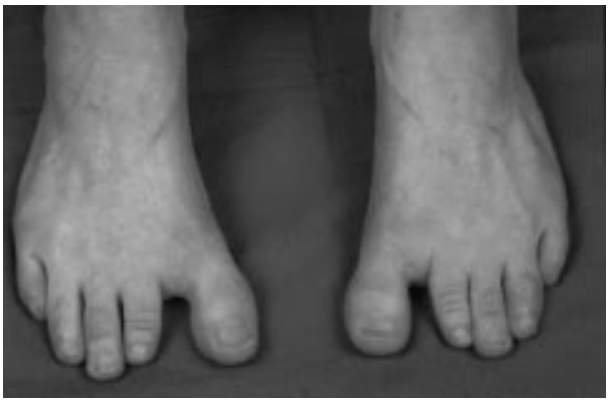

Figure 3 Feet of V.36 showing brachydactyly of the first, second, and fifth toes and a sandal gap between the first and second toes.

Figure 4 Hypospadias in one boy of the family: The urethral meatus is located distally on the penile shaft.

Nomenclature

Gene symbols used in this article follow the recommendation of the HUGO Gene Nomenclature Committee.[41] Mutations are described according to recommendations by den Dunnen and Antonarakis.[42]

RESULTS

Phenotype in affected family members

The family was originally identified because of the accumulation of hypospadias cases. Several phenotypic features distinguished this family from previously described families with HFGS (see table 1 for phenotype data and figs 2, 3, and 4 for photographs of affected family members). The limb abnormalities in this family were much milder and restricted to clinodactyly of the fifth finger and also several family members showed a varying degree of short thumbs. They also exhibited brachydactyly of the first, second, and fifth toe and a large gap between the first and second toe (a feature not previously described in HFGS). Affected family members had small feet with male shoe size less than 6.

X ray of the hands of three family members showed varying degrees of a malformed middle phalanx in the little finger

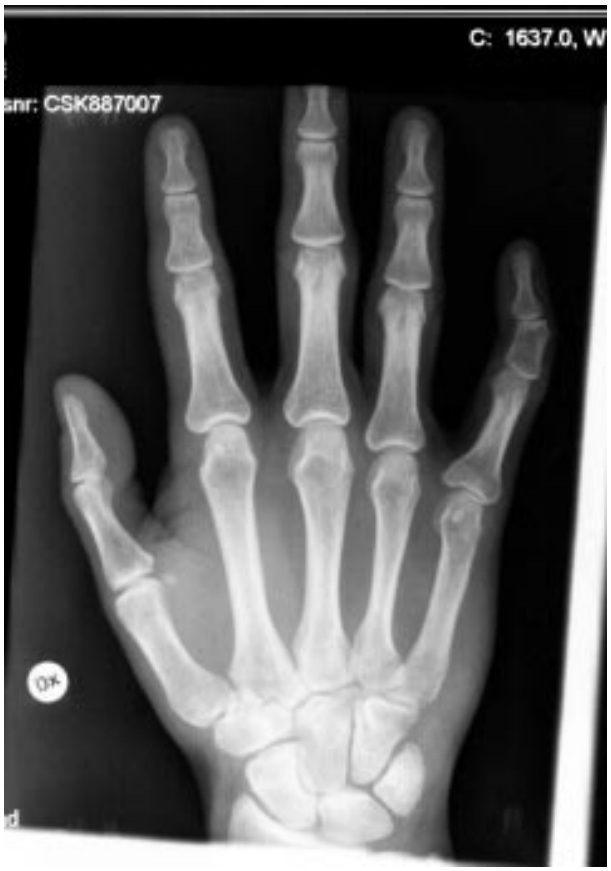

Figure 5 *X* ray of the right hand of V.35 showing a malformed middle phalanx in the little finger with a shorter radial surface.

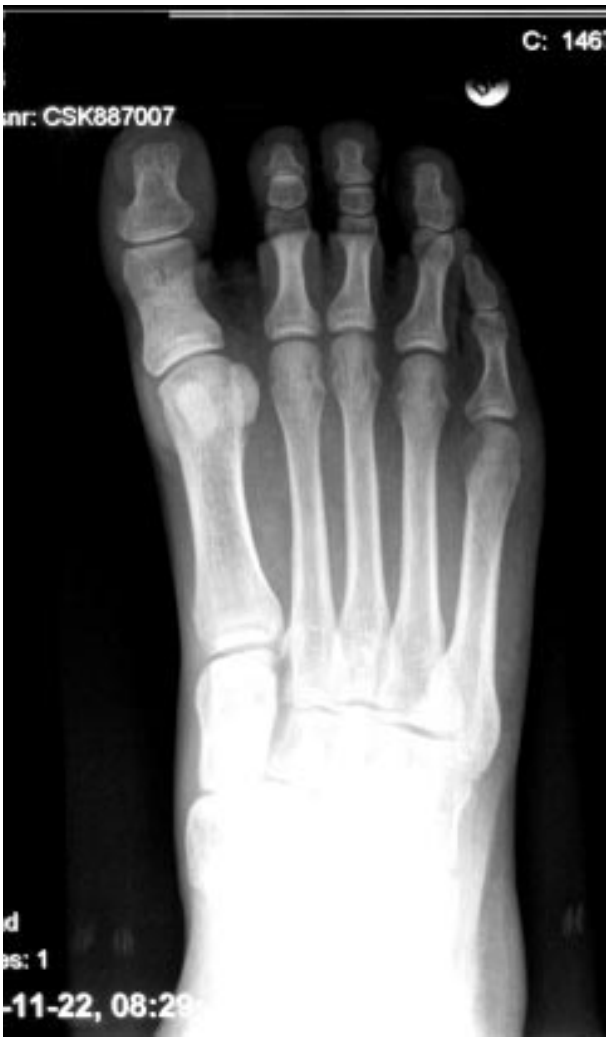

Figure 6 *X* ray of the right foot of V.35 showing a fusion between the two distal phalanges of the fourth and fifth toes, a sandal gap between the first and second toes, and also an indication of a short distal phalanx of the second toe.

with a shorted radial length causing clinodactyly. The most prominent findings in the feet were a fusion of the middle and distal phalanges of the fifth and sometimes also the fourth toe as well as a distinctly shortened end phalanx of the second toe (figs 5 and 6).

The urogenital abnormalities in affected females were restricted to urinary incontinence and recurrent urinary tract infections. Cystoscopy and voiding urethrocystography in VI.5 showed double kidneys on the right side and ectopic ureters ending in the bladder neck bilaterally. Two females (IV.11 and VI.5) had "short urethra" according to the medical case history. There were no reports of bicornuate uterus, vaginal septum, or female infertility. However, gynaecology records were only available for IV.11. Interestingly, there was a high penetrance of hypospadias in this family. All but two affected males had hypospadias. They were mildly affected with the urethra opening on the ventral side of glans, with the exception of three cases with proximal variants of hypospadias.

Mutation analysis

The genome wide linkage analysis showed evidence in favour of linkage to chromosome 7p. With additional markers we obtained a maximum lod score of 6.70 at $\theta=0$ for marker D7S503.

HOXA13 is located in this region at chromosome 7p15 (http://www.ncbi.nlm.nih.gov) and was therefore subject to mutation analysis. We used PCR primers amplifying the whole coding region of *HOXA13*. PCR and electrophoresis with the primers HOXA13-A65 and HOXA13-A66, amplifying part of exon 1, showed two alleles of different sizes in affected patients. Sequence analysis showed a duplication of 18 bp in the second polyalanine stretch in the first exon (234_251dup TGCGGCGGCGGCCGCGGC). This duplication was observed in all affected available family members (fig 1B). In addition, it was also found in V.18, who is apparently unaffected. The mutation was not found in 100 healthy subjects originating from Sweden, thus confirming that the mutation does not represent a polymorphism.

DISCUSSION

While searching for genes involved in hypospadias, we identified this family with atypical HFGS carrying a novel mutation in the *HOXA13* gene. The mutation consists of a duplication of 18 bp resulting in six additional alanines in the second polyalanine tract in the first exon of *HOXA13*.

This is the largest HFGS family reported so far, with 27 affected subjects in six generations. However, it appears to be an atypical variant of the syndrome since many phenotypic features distinguish this family from previously described HFGS families. The skeletal anomalies are less severe, in particular regarding the feet. Affected subjects have small feet, brachydactyly of the second and fifth toes, and a large gap between the first and second toes. The latter two features have not been described in any previously reported HFGS families; however, short or uniphalangeal second toes with absent nails are found in Guttmacher syndrome in which a mutation in the *HOXA13* gene was recently reported.[35 37] Hallux varus has only been reported in three subjects. Hypoplasia of the big toe, a

hallmark feature of HFGS found in all previously described families, is not present at all in this family. Moreover, there is no evidence of any Müllerian duct fusion defects in women, whereas in affected males there is a high penetrance of hypospadias. Interestingly, there is no obvious reduced fertility in this large pedigree. Since low birth weight is known to affect the risk for hypospadias,[2–5] we checked the birth weight of four affected males and they were all within normal limits. There is a striking variation in phenotype between affected subjects in this family, as reported previously in HFGS families. In the family described here, the skeletal as well as the urogenital abnormalities show variable expression. Interestingly, V.18 carries a mutation but claims to be unaffected. However, phenotype information could only be acquired by telephone interview and she declined further examination.

The polyalanine tract expansion in this family is stable through the generations (fig 1B). This is in line with the concept that cryptic polyalanine expansions may derive from unequal crossing over.[33] In the N-terminal region of *HOXA13*, there are three alanine repeats of 14, 12, and 18 respectively.[43] The insertion described here results in six additional alanines in the second polyalanine tract in the first exon, in contrast to the two previously reported polyalanine expansions (resulting in eight and six additional alanines, respectively) localised in the third tract.[23 26] It is likely that the discrepancies in phenotype between this and the previously reported families are caused by the different localisation of the insertion.

There are several reasons to question whether the malformations seen in patients heterozygous for the different mutations in *HOXA13* are the result of haploinsufficiency. The patient with a heterozygous deletion of the entire *HOXA* cluster has a relatively mild HFGS phenotype, in particular with regards to the genital malformations that are limited to cryptorchidism and chordee,[22] whereas the patient with a missense mutation has an unusually severe phenotype.[23] Mice heterozygous for a minor deletion within the *Hoxa13* gene have a more severe limb phenotype than mice homozygous for a *Hoxa13* null mutation.[44 45] The fact that heterozygous mutations in *Hoxa13* result in a phenotype more severe than anticipated from haploinsufficiency suggests that the mutant protein may not only be dysfunctional. It may instead have other deleterious effects, acting in a dominant negative way.

The mutations described in *HOXD13* may render a dominant negative protein as well, since the severity of synpolydactyly has been shown to correlate with the size of the polyalanine expansion.[30] Interestingly, in the family with the largest expansion (14 additional alanines) affected males have hypospadias. Urogenital manifestations (that is, lack of preputial glands in males) are also found in mice homozygous for a spontaneous polyalanine expansion (expanding the stretch from 15 to 22 alanines) in the *Hoxd13* gene.[46] Genetic complementation studies in this mouse model confirms that the mutated protein exerts a "super" dominant negative effect, by interfering with the function of the remaining wild type *Hoxd13* and other 5'*Hoxd* proteins.[47] Moreover, these mice have a much more severe phenotype than mice with complete absence of *Hoxd13* function.[46 48] Further evidence for dominant negative effects is derived from mice with homozygous deletions of *Hoxd11, Hoxd12*, and *Hoxd13* which have a less severe phenotype than mice and humans with polyalanine tract expansions in *HOXD13*.[46 48] The above observations are especially interesting in light of the suggested synergistic roles of *HOXA13* and *HOXD13*.[49]

In conclusion, we have described here a family with limb and urogenital malformations reminiscent, but yet distinct from those previously described in families with HFGS. The genetic basis for the phenotype in this family is a mutation in *HOXA13*. This finding illustrates the easily overlooked mild abnormalities resulting from mutations in human *HOX* genes.

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