CLINICAL STUDY

Mutations of CXorf6 are associated with a range of severities of hypospadias

Nicolas Kalfa[1,2], Benchun Liu[1], Klein Ophir[3], Francoise Audran[2], Ming-Hsieh Wang[1], Cao Mei[1], Charles Sultan[2] and Laurence S Baskin[1]

[1]*Department of Pediatric Urology, Center for the Study and Treatment of Hypospadias, Children's Medical Center, University of California San Francisco, 400 Parnassus Avenue, A 640, San Francisco, California 94143, USA,* [2]*Service d'Hormonologie, Hôpital Lapeyronie, CHU Montpellier, Montpellier, France and* [3]*Department of Orofacial Sciences and Pediatrics, Institutes of Human Genetics and Regeneration Medicine, University of California San Francisco, San Francisco, California, USA*

(Correspondence should be addressed to L S Baskin; Email: lbaskin@urology.ucsf.edu)

Abstract

Objective: Mutations in *chromosome X open reading frame 6* (*CXorf6*), a recently described candidate gene involved in the development of male genitalia, have been found in patients with complex 46,XY disorders of sexual development (46,XY DSD) including micropenis, bifid scrotum, and penoscrotal hypospadias. The objective of this work was to identify genomic variants of *CXorf6* in patients with isolated hypospadias, severe or non-severe.

Design and methods: Forty-one patients with glandular to perineal hypospadias and thirty controls were studied. Direct sequencing for coding exons 3–6 of *CXorf6* and their flanking splice sites was performed on DNA extracted from foreskin collected from surgery. Secondary and tertiary structures of the protein were predicted using NNpredict and Protein Homology/analogY Recognition Engine engines.

Results: Four mutations (9.7% of cases) were identified. One missense mutation (1295T>C, V432A) and two deletions (325delG, predicted to cause a stop codon L121X) occurred in patients with penoscrotal and proximal hypospadias. One patient with subcoronal hypospadias had CAG-repeat amplification in the second polyglutamine domain of *CXorf6*. Secondary structure prediction indicated that this insertion occurred in a helix element of the protein. The tertiary structure prediction showed an alteration of the shape of the protein and crowding between domains.

Conclusion: *CXorf6* mutations are associated with isolated hypospadias of varying severity. However, the pathophysiology of these mutations and the function of the *CXorf6* gene product remain to be investigated.

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Introduction

Hypospadias occurs in 1 out of 300 live male births (1). Approximately, 6000 boys with hypospadias are born in the United States each year (2). There is a 14% incidence in male siblings and 8% incidence in offspring of males affected with hypospadias (3). Normal penile and urethral development begins in the sixth week of gestation with the formation of the urogenital sinus, which eventually becomes masculinized under the direction of testosterone and its more potent form dihydrotestosterone. Without the presence of adequate levels of testosterone (4) or a functioning androgen receptor (5), the genital structures become female in appearance, as seen in the most severe cases of hypospadias. The overwhelming majority of cases of hypospadias remain unexplained, particularly the milder forms. Any new gene implicated in differentiation of genitalia is therefore a candidate gene as a possible etiology for hypospadias.

One of the most recent candidate genes identified for the development of the male genitalia is *CXorf6* (formerly F18). This gene, discovered in the course of identifying the gene responsible for X-linked myotubular myopathy, *MTM1*, maps to proximal Xq28 (6, 7). No significant homology to other known proteins was detected, but segments of the first open reading frame encode polyglutamine tracts and proline-rich domains that are frequently observed in DNA-binding proteins. Northern blot analysis of *CXorf6* mRNA showed ubiquitous expression that was particularly high in skeletal muscle, brain, and heart (7). It is also hypothesized to be implicated in male genital development. Indeed, myopathic individuals with intragenic mutations of *MTM1* have normal sexual development, whereas those with microdeletions of *MTM1* extending to the *CXorf6* locus have abnormal genitalia (8–11). Subsequent studies have demonstrated that *CXorf6* is mutated in 46,XY disorders of sexual development (46,XY DSD): Fukami *et al*. (12) have identified three nonsense mutations in four individuals with 46,XY DSD including micropenis, bifid scrotum, and penoscrotal hypospadias.

The aims of this study were to characterize the genotype of *CXorf6* in patients with various types of isolated hypospadias and to evaluate the incidence of polymorphisms of *CXorf6* in this frequent malformation.

Materials and methods

Patients and controls

In this study, 71 individuals (from newborn to 6 years) were included. Forty-one patients presented with isolated hypospadias or familial history of genital malformation. Clinical severity ranged from glandular to perineal hypospadias. Patients were characterized as 'severe' and 'non-severe' groups. Figure 1 summarizes the patients' phenotypes. Abnormalities in androgen biosynthetic pathway were unlikely in these boys with isolated hypospadias without accompanying cryptorchidism or micropenis as shown in our previous study using adrenocorticotrophin test (13). Thirty individuals who underwent a circumcision were included as controls. This study was approved by the Institutional Review Board and written consent was obtained. When a mutation was identified, other family members were examined when possible. Regarding ethnicity of patients, the study and control groups were mixed (Caucasian, Asiatic, Afro-American).

DNA extraction

Excess skin at the time of hypospadias surgery and/or circumcision was frozen in liquid nitrogen. DNA was extracted from this tissue using DNAzol (Invitrogen). The manufacturer's protocol for DNA isolation was followed with minor modifications.

Mutational analysis

Direct sequencing of coding exons of *CXorf6* and their flanking splice sites was performed. Primers are summarized in Table 1. The 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) was used. Sequencing reactions were repeated twice with at least two different PCR products. The DNA sequences were compared with the sequences of normal controls and to the reference genome from the ensembl.org database and genebank database (GenBank accession number NM_005491).

Structure prediction

Secondary structure was predicted with NNpredict, University of California, at http://alexander.compbio.ucsf.edu/~nomi/nnpredict.html based on homology sequence. The three-dimensional structure was predicted at Protein Homology/analogY Recognition Engine (PHYRE) from the Structural Bioinformatics Group, Imperial College, London, at http:www.sbg.bio.ic.ac.uk/phyre~/. Briefly, this program, developed and previously tested extensively by Kelley *et al.* (14), combines the power of multiple sequence profiles with structure-based profiles (9, 14). This method uses structural alignments of homologous proteins of similar three-dimensional structure in the structural classification of protein databases to obtain a structural equivalence of residues. It can detect remote homologous proteins with similar tertiary structures, especially with respect to the active site, by comparing their three-dimensional position-specific scoring matrix, even though they have low similarity between their amino acid sequences (15). On the other hand, this tool is not designed to assess the consequences of point mutations and was not used as such in this work.

Molecular analysis of the androgen receptor gene

To exclude a defect of androgen sensitivity in patients where we identified a genomic variant of *CXorf6*, we performed a molecular analysis of the androgen receptor
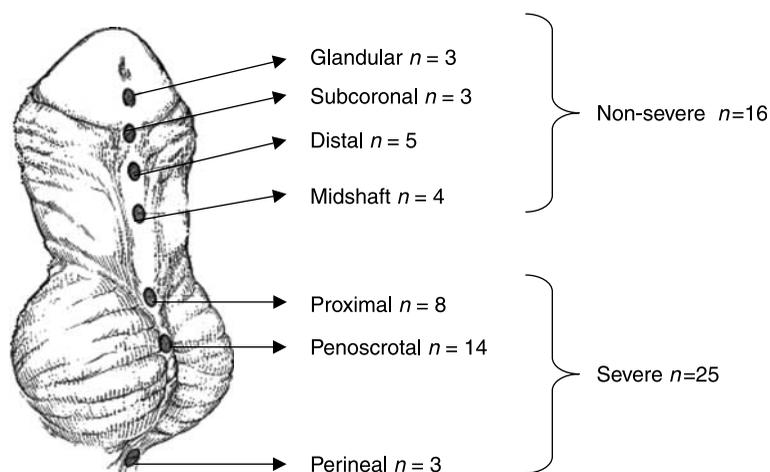
Figure 1 Summary of the patients' phenotypes. Clinical severity ranged from glandular to perineal hypospadias. Patients were characterized as 'severe' and 'non-severe' groups.

Table 1 Primers used for exons 3–6 amplifications.

	Forward	Reverse	Size of PCR product (bp)
Exon 3			
Part 1	CTCTCTTCTCCTCTTCTCTTCTCT	GGTGAGCTCCTCTAGCAGCTCTTG	416
Part 2	GCCGGCTGTAGACCAGGAGC	GGGCGTGATGCCACTGAGGC	407
Part 3	GTCGCTCTGCCCCCCTTACC	GAATGGAGCTCTGAGGGCCG	412
Part 4	GCTCTCACTCAACAGCCGCAG	GGCTCCGTGGCTCCTGGGCTG	368
Part 5	CATGATCATGCAGCAGGGGATGGCAAGC	CCAAAACAAAAGAACACAGCCAGATATG	401
Exon 4	TAGGACACGGCAGGCCACCTG	AGCCAGCTGATCCAGCTCTGG	282
Exon 5	TTTGTGGCCAAGCAGCTGATG	GACTGTGCCCCGTTGACAGAT	342
Exon 6	CTAGGTCCCCACGCAGCGATC	ACCCCTTGCCCACCCCTTTGG	210

The cycling protocol was identical for all exons and consisted of an initial denaturation step at 93 °C (2 min), followed by 39 cycles of denaturation at 93 °C (30 s), hybridization at 58 °C (30 s), and extension at 72 °C (30 s), with a final extension step of 6 min at 72 °C. To ensure the absence of contamination, DNA from controls and DNA-free samples were run simultaneously. All PCR products were analyzed on a 2% agarose gel. DNA sequencing was performed with the antisense and/or sense primer.

gene. Exons 1–8 of the AR were amplified by PCR using sets of primers and reactions previously described (16). PCRs were verified for correct length on agarose gel, purified with Qiaquick PCR columns (Qiagen), and sequenced with the ABI Prism Big Dye terminator sequencing kit and the ABI 310 genetic analyzer (Applied Biosystems, Courtaboeuf, France).

Results

Three different mutations were found in four non-related patients out of the 41 patients with both severe and non-severe hypospadias (9.7%). None of these mutations were noted in the control group. No mutation of the androgen receptor gene within exons 1–8 was identified in these patients.

Three mutations of *CXorf6* occurred in patients with severe hypospadias. The 1295T>C (V432A) was found in a patient with a proximal hypospadias (Case 1, Fig. 2), but the mother of this family could not be studied and there was no family history of hypospadias. Two deletions at the beginning of the first translated exon were also identified (325delG, Fig. 2). This deletion induced a shift in the reading frame from amino acid 109 and was predicted to induce an early stop codon at amino acid 121
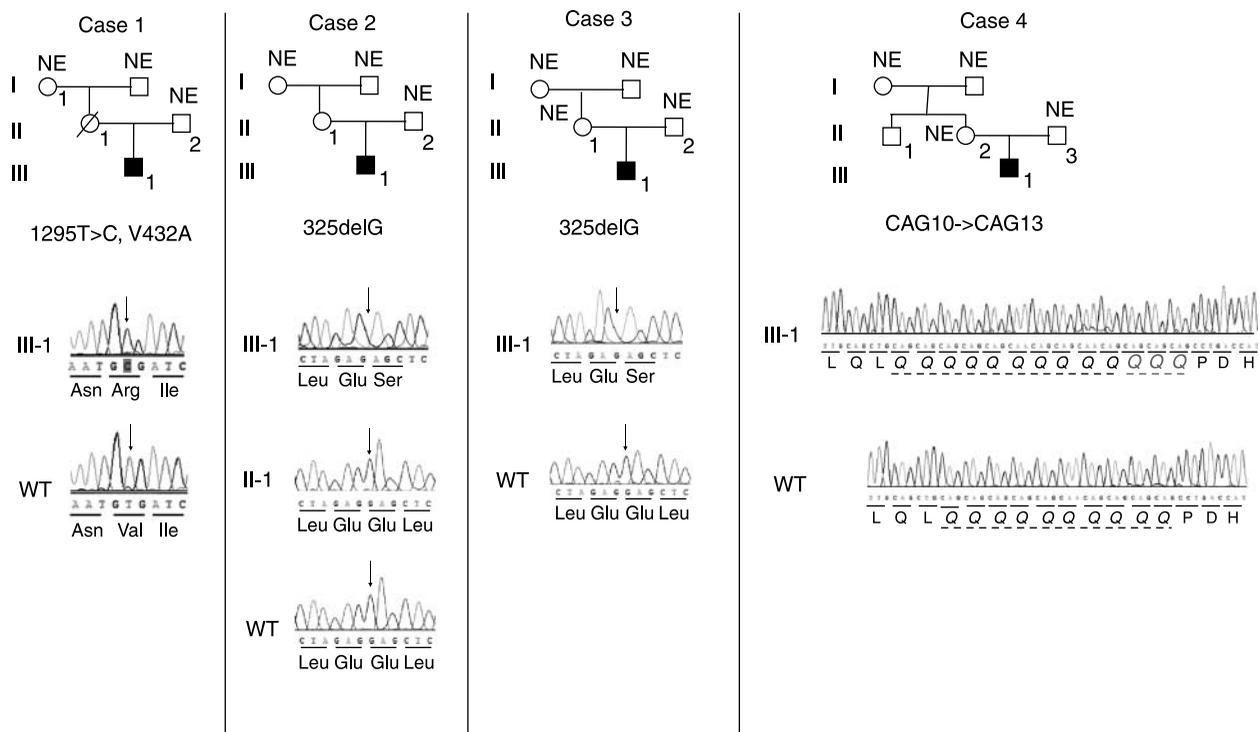
Figure 2 Electrochromatograms and pedigrees of the four patients with mutations of *CXorf6*. NE, not examined; the black squares indicate patients with hypospadias. All mutant sequences were controlled by a wild-type (WT) DNA.

(E109fsX121) and thus a short truncated protein. The phenotypes of these patients were a proximal hypospadias (case 2) and a penoscrotal hypospadias (case 3). One of the mothers of these two families did not have any heterozygous mutation. The other one was not available for study. Again there was no familial history of hypospadias.

One mutation occurred in a patient with a non-severe hypospadias. An insertion of the second polyglutamine domain of the protein was found in a boy with an isolated subcoronal hypospadias ($CAG_{10} \rightarrow CAG_{13}$). This insertion of 9 nucleotides, which encodes 3 additional glutamine residues, increased the length of this domain from 10 to 13 residues (case 4, Fig. 2). The secondary structure predicts that this insertion occurs in, or close to, a helix element of the protein (data not shown). The tertiary structure prediction, performed despite a relatively low homology of CXorf6 to other known proteins, was in accordance with this result and showed an alteration in the shape of the protein (Fig. 3a) and crowding between domains (Fig. 3b). Blood from the mother was not available. There were no related cases of hypospadias in the family. This variation of the length of the polyglutamine domain was not observed in the control group. Clinical and hormonal data of patients with mutated CXorf6 are summarized in Table 2.

Discussion

Direct evidence for the implication of *CXorf6* in 46,XY DSD without MTM was recently reported by Fukami *et al.* (12). Three nonsense mutations (E124X, Q197X, and R653X) were identified in four Japanese individuals with penoscrotal hypospadias, chordee, micropenis, bifid scrotum, and a case of hypoplasic scrotum with inguinal testis. Here, we report that *CXorf6* mutations are also found in patients with a spectrum of hypospadias phenotypes, from penoscrotal to glandular variety. These genomic variants were absent from our normal patients and were not reported in the group of 150 controls by Fukami either (12). Our findings suggest that i) mutations associated with hypospadias occur more frequently on exon 3, ii) insertion of nine nucleotides coding for three additional glutamine may be involved in causing hypospadias, and iii) polymorphisms in the gene may play a role in hypospadias.

We found that the occurrence of an early nonsense codon in *CXorf6* is frequently associated with hypospadias. The 325delG mutation and the subsequent frameshift and stop codon at the beginning of exon 3 was found in patients with proximal or penoscrotal hypospadias. This position is predicted to cause nonsense-mediated mRNA decay (17). Our finding is in accordance with the previous literature. Fukami *et al.*
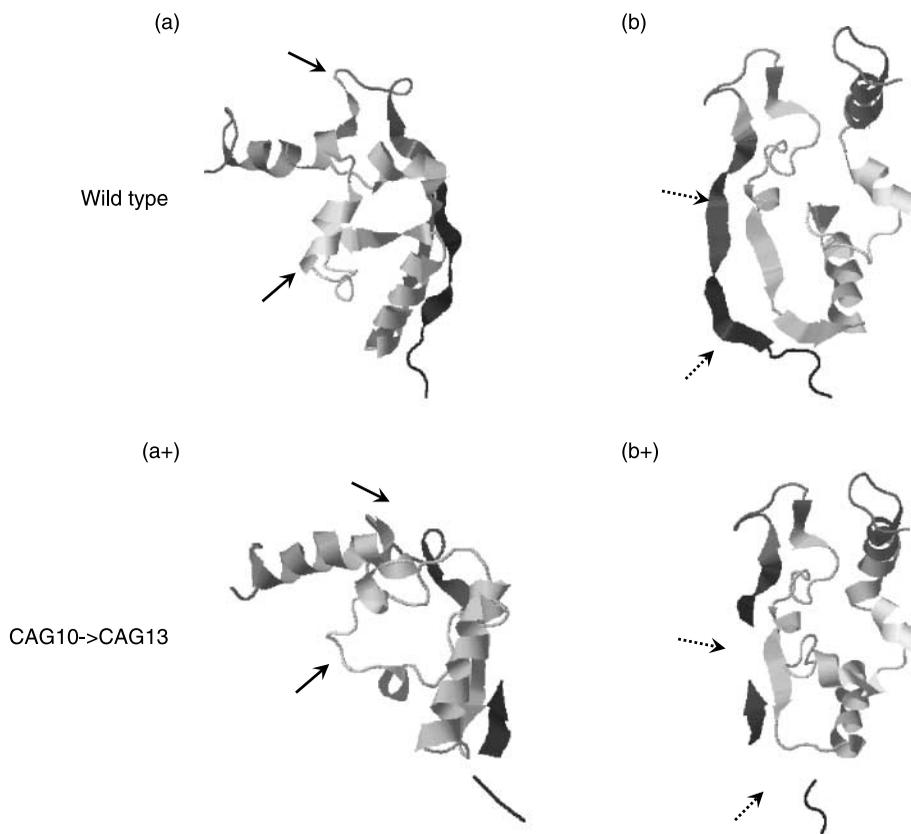
Figure 3 Tertiary structure prediction of the wild-type protein (3a and 3b) and with the CAG-repeat amplification (3a+ and 3b+). The three-dimensional structure was predicted at Protein Homology/analogY Recognition Engine (PHYRE) from the Structural Bioinformatics Group, Imperial College, London, at http:www.sbg.bio.ic.ac.uk/phyre ~ /. The plain arrows show the changes in the shape of the protein between the wild-type (3a) and the CAG-repeat amplification (3a+). The broken arrows show crowding between domains (3b+) that do not exist in the wild-type protein (3b).

Table 2 Clinical and hormonal data of patients with mutated CXorf6.

Patient	Case 1	Case 2	Case 3	Case 4
Previous medical history	None	Prematurity and low birth weight[a], Necrotizing enterocolitis, Asthma, ADHD[b]	Low birth weight[c], bilateral cataracts operated at 3-years old	None
Genital phenotype				
Diagnosis	Isolated hypospadias	Hypospadias	Hypospadias with chordee	Isolated hypospadias
Position of meatus	Penile shaft, proximal	Penile shaft, proximal	Penoscrotal	Coronal
Age at exam (yr, mo)	5,06	1,00	1,02	0,01
Micropenis	No	No	No	No
Testis position	Intrascrotal	Inguinal	Intrascrotal	Intrascrotal
Testis size (normal = 1–2 ml)	Normal	Normal	Normal	Normal
Scrotal appearance	Normal	Normal	Normal except at the area of the ectopic urethral meatus	Normal
Renal structure	Normal	Normal	Normal	Normal
Extragenital phenotype	Normal	Normal	Normal	Normal
Growth				
Present age (yr, mo)	8, 6	8, 1	1, 1	NA
Present height (cm)	131 (+0.5)	117 (−1.5)	75 (0)	NA
Present weight (kg)	25 (0)	20.9 (−1)	9.7 (−0.5)	NA
Serum hormone level				
Age at the exam (yr, mo)	ND	1, 2	1, 2	ND
Testosterone (ng/dl)	ND	<2 (<13)	5 (<13)	ND
DHT (nmol/l)	ND	<2 (<3)	2.1 (<3)	ND
Androstenedione (ng/dl)	ND	17 (5–51)	15 (5–51)	ND
DHEA (ng/dl)	ND	91 (20–110)	106 (20–110)	ND
Progesterone (ng/dl)	ND	<5 (4–52)	<5 (4–52)	ND
17 Hydroxyprogesterone (ng/dl)	ND	59 (42–540)	ND	ND
17 Hydroxypregnenolone (ng/dl)	ND	76 (15–221)	78 (15–221)	ND
Cortisol (μg/dl)	ND	11 (5–20)	7 (5–20)	ND
11 Deoxycortisol (ng/dl)	ND	41 (20–155)	ND	ND

ADHD, attention-deficit hyperactivity disorder; SD, s.d.; ND, non-determined; NA, not available; DHT, dihydrotestosterone; DHEA, dihydroepiandrsosterone. Brackets indicate the s.d. for height and weight, and normal range for hormone serum levels.
[a]Thirty weeks of gestation, birth weight 1.2 kg.
[b]CXorf6 is expressed in the brain.
[c]Full term, birth weight 2.4 kg.

described two nonsense mutations in the same exon associated with DSD (12). Tsai *et al.* also suggested that exon 3, which encodes 80% of the protein, is required for normal CXorf6 function (18). These authors reported a case of chimeric CXorf6–MTMR1 fusion transcript including exon 3 in a child without genital malformation. Similarly, we did not find any nonsense mutations of exon 3 controls.

Congenital malformations associated with insertion of several glutamine are rare, particularly in the urogenital system. The only previous report concerns the polyglutamine tract of androgen receptor in patients with undermasculinized genitalia, which is significantly longer than that in the control group (19) but there is no evidence for the polyglutamine stretch expansion being the causative factor for hypospadias. Even if such an insertion can function as a susceptibility factor for the development of hypospadias, the role of CAG insertion of CXorf6 found in our study is still unclear. The function of this protein remains unknown and, even if the tertiary structure of the protein appeared to

be modified, the negative effect of this amplification remains to be demonstrated.

The mechanism by which *CXorf6* mutations induce hypospadias remains to be elucidated. Because defective testicular androgen production or responsiveness is known to lead to hypospadias, CXorf6 was first suspected to impair the production of androgen. Previous studies indicated that CXorf6 is expressed in fetal Sertoli and Leydig cells during the critical period for sex development (12, 20). Moreover, *CXorf6* augments testosterone production and contains the steroidogenic factor 1 (SF1) target sequence (21). A transient Leydig cell dysfunction has thus been hypothesized to explain both the *in utero* under-masculinization and the normal post-natal plasmatic testosterone level as confirmed in our study. Another hypothesis is that CXorf6 mutations may cause placental malfunction that leads to hypospadias. An alternative form of CXorf6 of 3.8 kb is indeed expressed in the placenta (7). In addition, early placental malfunction – and subsequent low birth weight as seen in two of our patients – has been

previously suspected to increase the risk for hypospadias (22, 23). Finally, the postnatal hormonal parameters and the testicular clinical exam were normal for our patients, suggesting an intrauterine defect rather than abnormal testicular development.

The data from this study indicate that genetic variants of *CXorf6* are present in patients with isolated hypospadias of varying severity. The function of CXorf6 remains nevertheless to be elucidated.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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