

Mutation Analysis of *FOXF2* in Patients with Disorders of Sex Development (DSD) in Combination with Cleft Palate

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Key Words

Cleft palate · Disorders of sexual development · *FOXF2* · Genital development · Genital tubercle

Abstract

In contrast to disorders of sexual differentiation caused by lack of androgen production or inhibited androgen action, defects affecting development of the bipotent genital anlagen have rarely been investigated in humans. We have previously documented that the transcription factor *FOXF2* is highly expressed in human foreskin. Moreover, *Foxf2* knock-out mice present with cleft palate in combination with hypoplasia of the genital tubercle. We hypothesized that humans with disorders of sex development (DSD) in combination with cleft palate could have mutations in the *FOXF2* gene. Eighteen children with DSD and cleft palate were identified in the Lübeck DSD database (about 1,500 entries). Genomic DNA sequence analysis of the *FOXF2* gene was performed and compared with 10 normal female and 10 normal male controls, respectively. Two heterozygous DNA sequence variations were solely present in one single patient each but in none of the 20 normal controls: a duplication of

GCC (c.97GCC[9]+[10]) resulting in an extra alanine within exon 1 and a 25*G>A substitution in the 3'-untranslated region. Two patients carried a c.262G>A sequence variation predicting for an Ala88Thr exchange which was also detected in 2 normal controls. Two silent mutations, c.1272C>T (Ser424Ser) and c.1284T>C (Tyr428Tyr), respectively, occurred in the coding region of exon 2, again in both patients and normal controls. In conclusion, the majority of the detected sequence alterations were polymorphisms without obvious functional relevance. However, it cannot be excluded that the 2 unique DNA sequence alterations could have affected *FOXF2* on the mRNA or protein level thus contributing to the observed disturbances in genital and palate development.

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Human sexual development can be divided into sexual determination and sexual differentiation. Sex determination comprises development of the bipotent gonadal anlagen into either testes or ovaries (Hiort and Holterhus, 2000). Sexual differentiation comprises development of the internal and external genital organs under the control of sex hormones, i.e., the presence or absence of tes-

tosterone and Anti-Müllerian hormone (AMH) (Hiort and Holterhus, 2000). Disorders of sexual development (DSD) may occur at every single step due to interruption of the normal genetic and hormonal pathways leading to lack of androgens because of gonadal dysgenesis, defects in androgen biosynthesis, or inhibited androgen action in 46,XY males, eventually resulting in defective external masculinization.

A lot of children born with ambiguous genitalia lack a definite diagnosis because the mechanisms of normal and abnormal genital development in humans are still poorly deciphered. Interestingly, many DSD children present with associated malformations (Thyen et al., 2006). This suggests that so far unknown genetic events may interfere with specific early stages during embryogenesis potentially affecting development of both the genitalia and distinct somatic tissues. In a previous cDNA microarray based genome-wide study we identified that the transcription factor *FOXF2* was significantly overexpressed in human foreskin fibroblasts from normal male individuals compared to labia majora fibroblasts from DSD patients with androgen insensitivity syndrome (Holterhus et al., 2003). Moreover, *FOXF2* showed an almost identical expression pattern as *HOXA13* (Holterhus et al., 2003) known to cause the hand-foot-genital syndrome, a combination of limb malformations and genital abnormalities (Mortlock and Innis, 1997). The *FOXF2* gene belongs to the growing family of forkhead-box transcription factors characterized by a highly conserved ~100-amino acid DNA binding domain. The human *FOXF2* gene consists of 2 exons divided by an intron and is located at 6p25.3 (Blixt et al., 1998). Forkhead-box transcription factors play important roles in many aspects of embryonic development (Kaufmann and Knöchel, 1996). *Foxf2* shows an early expression in the head mesenchym adjacent to the oropharynx and stomodeum during mouse embryogenesis (Ormestad et al., 2004), later on also in the respiratory system, urinary system, skeletal system, as well as the eye and inner ear (Aitola et al., 2000). Homozygous mutant *Foxf2* knockout mice presented with cleft palate and an abnormal tongue and died shortly after birth (Wang et al., 2003). As described later, *Foxf2* knockout mice also showed abnormalities of the external genitalia with a significant hypoplasia of the genital tubercle. We hypothesized that *FOXF2* gene mutations could play a role in children with the combination of abnormalities of the external genitalia and cleft palate. In this study, we report the genomic DNA sequence analysis of the *FOXF2* gene in DSD patients combined with cleft palate.

Materials and Methods

The study was approved by the ethical committee of the University of Lübeck, Germany.

Patients and Normal Controls

Eighteen children were selected from 1,500 cases of the Lübeck DSD database because of the clinical combination of DSD and cleft palate. Table 1 summarizes the clinical, cytogenetic, molecular genetic, and hormonal findings. Ten normal female and 10 normal male individuals served as controls.

Genomic DNA Analyses

Genomic DNA was extracted from blood leukocytes according to standard procedures. The whole coding region of the *FOXF2* gene including the exon-intron junctions was amplified by PCR. Nine primer pairs were selected using the software PrimerSelect 6.00 (DNASStar Inc. Madison, Wisconsin, USA) and they are listed in table 2. Exon 1 consists of 1,170 bp and was amplified in 8 overlapping fragments. Exon 2 was amplified as a whole. Amplification was performed on a programmable thermocycler (PTC-2000, My Research, Inc., Massachusetts, USA) using 100 ng of DNA in a 50 μ l mixture including 20 pmol of each primer, 2 mM dNTPs (dATP, dGTP, dTTP, and dCTP), 1 U DNA-Polymerase (AmpliTaq, Roche), 20 mM Tris (pH 8–8.8), and 1.0–2.0 mM $MgCl_2$. Exon 1 contains GC-rich areas. Therefore, to receive specific fragments, 5% DMSO was added for amplifying exon 1. Starting with an initial denaturing at 94°C for 5 min, the PCR program consisted of 34 cycles, denaturation at 98°C for 10 s, annealing at 49–66°C for 30 s, and elongation at 72°C for 2 min, and ended by a final elongation at 72°C for 5 min.

For mutation screening, non-isotopic SSCP analysis (single strand conformation polymorphism analysis) was used. The fragment 1/6 of exon 1 was digested using the restriction enzyme *PvuII* prior to SSCP. PCR products were diluted 1.5:1 in 95% formamide, 89 mM Tris, 20 mM EDTA, 89 mM boric acid, 0.05% bromophenol blue, and 0.05% xylene cyanol. After a 5-min denaturation at 98°C, the PCR products were chilled on ice water and afterwards loaded onto a 6% polyacrylamide gel (0.8 mm thick) containing 5–10% glycerol. Electrophoresis was performed at 8–40 W for 4–17 h at room temperature. DNA bands were visualized using silver staining (Bassam et al., 1991). All fragments with aberrant mobility (band shifts) were sequenced using an automatic capillary sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA) according to standard procedures. In case of band shifts, the same PCR product was analysed in the 20 control samples as well. Since some exon 2 samples showed only minimal aberrations in migration patterns, this exon was directly sequenced in all 18 patients without prior SSCP.

Results

Previously we found that *Foxf2*^{-/-} mice showed cleft palate (Wang et al., 2003). Recently, we also found that the genital tubercle was hypoplastic in these homozygous mice (fig. 1). Therefore, the *Foxf2* knockout mice dis-

Table 1. Summary of the patients

Patient	Karyotype	External genitalia	Facial cleft	Associated malformations	Analysed genes without mutations	Hormone levels ^a
1	46,XY	micropenis, scrotum bipartitum, hypospadias	cleft palate	multiple anomalies including wide Aorta descendens, macrocephaly, hypertelorism, mental retardation	<i>AR, SRY</i>	testosterone basal: 0.285 nmol/l
2	46,XY	micropenis, hypospadias	cleft palate	broad thumbs, prominent forehead, hypertelorism, dysplastic teeth and ears	<i>AR</i>	T baseline: 0.451 nmol/l, stimulated: 3.157 nmol/l; DHT: 0.7912 nmol/l; A: 2.094 nmol/l (age 5 years) [0.07–0.31 nmol/l]
3	46,XY	scrotum bipartitum, perineal hypospadias	cleft palate	mental retardation, microcephaly, epilepsy, growth delay, iris coloboma	<i>AR</i>	T baseline: 0.662 nmol/l, stimulated: 5.812 nmol/l
4	46,XY	hypospadias	cheilognathopalatoschisis, cleft larynx	growth delay, epicanthus, broad nasal root	<i>AR, 5α-reductase II</i>	T baseline: <0.7 nmol/l; DHT: 0.6536 nmol/l
5	46,XX	uterus duplex, vaginal agenesis	cheilognathopalatoschisis	aplastic kidney on the right, double-kidney on the left side, Meckel's diverticulum	<i>AR, SRY</i>	T baseline: 0.062 nmol/l (age 3 years) [0.07–0.35 nmol/l]
6	46,XY	scrotum bipartitum, hypospadias	cleft palate with bifid uvula		<i>AR, 5α-reductase II</i>	T baseline: 0.937 nmol/l, stimulated: 7.388 nmol/l; DHT: 0.25 nmol/l
7	46,XY	micropenis, scrotal hypospadias	cleft palate	retrognathia, hypoplastic left kidney	<i>AR, 5α-reductase II</i>	
8	46,XY	micropenis	cheilognathopalatoschisis	deafness	no information	T baseline: <0.7 nmol/l
9	46,XY	micropenis, hypospadias	cheilognathopalatoschisis	Falot's syndrome, iris coloboma	<i>AR, 5α-reductase II</i>	not documented
10	46,XY	scrotal hypospadias	cleft palate	growth delay, facial abnormalities, vitium cordis	<i>AR</i>	not documented
11	46,XY	hypospadias, scrotum bipartitum	cleft palate	hypoplastic rib, premature closure of the sagittal suture	<i>AR, 5α-reductase II</i>	not documented
12	not documented	micropenis, cryptorchidism	cheilognathopalatoschisis	esophageal atresia Vogt IIIb, mental retardation, vitium cordis	<i>AR: silent mutation, 5α-reductase II</i>	not documented
13	46,XY	hypospadias, cryptorchidism	cleft palate	mental delay, congenital hypothyroidism	<i>AR, 5α-reductase II</i>	not documented
14	46,XY	perineal hypospadias, scrotum bipartitum, septal urorectale	cheilognathopalatoschisis	hypertelorism, anal atresia, iris coloboma, dysplastic left kidney, accessoric left mamilla	no information	not documented
15	46,XY	ambiguous genitalia	cheilognathopalatoschisis		<i>AR, 5α-reductase II</i>	T baseline: 0.382 nmol/l, stimulated: 8.502 nmol/l; DHT: 0.206 nmol/l; A: 1.152 nmol/l
16	46,XY, partial trisomy 3q	scrotal hypospadias, ambiguous genitalia with persistent vaginal anlagen	cleft palate	vitium cordis with ventricular septal defect, right sided aortic arch	<i>AR</i>	T baseline: 0.07 nmol/l, stimulated: 9.404 nmol/l (age 16 months) [0.07–0.35 nmol/l]
17	45,X/46,XY	unclassified pseudohermaphroditism	cleft palate		no information	T baseline: 0.729 nmol/l, stimulated: 3.435 nmol/l; A: 2.792 nmol/l (age 7 years) [0.07–0.38 nmol/l]
18	not documented	hypospadias	cheilognathopalatoschisis	pyelectasis	no information	not documented

^a (age at determination if documented) [normal *baseline* range of T related to age]; T: testosterone; DHT: dihydrotestosterone; A: androstenedione; normal stimulated testosterone in the hCG test >3.5 nmol/l (>100 ng/dl); normal stimulated T/DHT ratio in the hCG test <16; normal A/T ratio <1.

Table 2. Primers used for amplification of *FOXF2*. S: sense, A: antisense.

Fragment	Primer
Exon 1/1	S: 5'-GGC GCT CGC AGG GCT TCT-3' A: 5'-TCA TCA GGG CGG CCT GGA-3'
Exon 1/2	S: 5'-CCG GGT CCC AGA TGA CCA-3' A: 5'-GGG GCG CTG GCC GAA TTG-3'
Exon 1/3	S: 5'-GTC GTC GTC CTC CGC CTC CT-3' A: 5'-TTG CTG GGC GAG CTC TGG AT-3'
Exon 1/4	S: 5'-CTA CTC GTA CAT CGC GCT CAT-3' A: 5'-GAG GCC CTT AGG CAG CTT G-3'
Exon 1/5	S: 5'-AGG GCT GGA AGA ACT CGG T-3' A: 5'-TAC ATG GGC TTG AGC GCC T-3'
Exon 1/6	S: 5'-CGA CCC GGC CAG CGA GTT CAT-3' A: 5'-GGT ACC GGG CTG CTG CTG TCC-3'
Exon 1/7	S: 5'-CAG CCA CGC GCA CCC TCA-3' A: 5'-GCG AGT AGG AGG ACA TGC TGG A-3'
Exon 1/8	S: 5'-GCG CCA TCG AAT GCC ACT-3' A: 5'-AGC CCT GGA GGC CTA GAA-3'
Exon 2	S: 5'-CTG GAT GAC TTT GTT TCT GA-3' A: 5'-CGT GCA TGT GAC TTG AAT-3'

played both hypoplastic genitalia and cleft palate. The similarity of the DSD patients combined with cleft palate with the phenotypes of *Foxf2* knockout mice prompted us to study the *FOXF2* gene in DSD patients with cleft palate.

In patient 10 (table 3, fig. 2) we detected a heterozygous insertion of an additional GCC repeat (c.97GCC[9]+[10]; accession no. ss105106973) predicting an insertion of an extra alanine in a polyalanine stretch within the N-terminus of *FOXF2*. This DNA sequence variation was neither detected in any of the 20 normal controls nor in any other patient and has not previously been described. In contrast, we found the heterozygous sequence variation c.121G>T affecting the same alanine repeat predicting for an A41S exchange on the protein level only in one normal female control but not in any of the patients. The affected alanine is the last residue of the 9 residues long polyalanine repeat.

Two additional novel SNPs were detected in the coding region of *FOXF2* during our analyses. Two patients (patients 3 and 14) as well as 2 male controls showed a heterozygous nucleotide substitution c.262G>A (accession no. ss107795095) predicting a conversion of alanine to threonine at codon position 88 (A88T) (table 3, fig. 2).

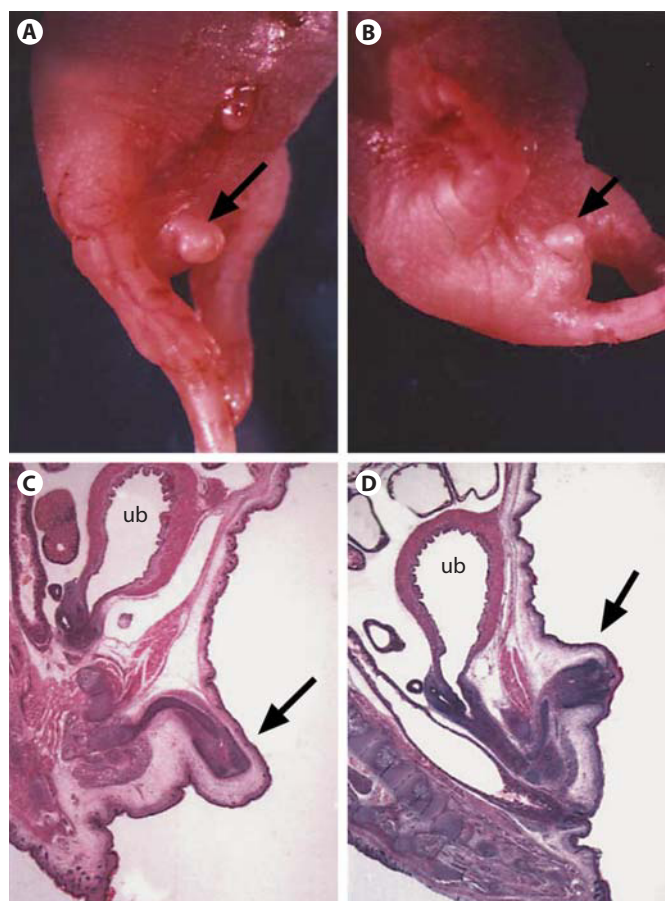


Fig. 1. External genitalia of a normal control mouse and a *Foxf2*^{-/-} knockout mouse at day P0 (postnatal day 0). **A** Macroscopic aspect of a normal control mouse; **B** *Foxf2*^{-/-} mutant mouse depicting hypoplasia of the genital tubercle; **C, D** Underlying histological findings. The genital tubercle is marked by an arrow. ub = urinary bladder.

The second polymorphism was a silent mutation in the coding region of exon 2. A heterozygous exchange of cytosine by thymine at position 1272 (c.1272C>T; S424S; accession no. ss105106972) was found in patient 15 and in 1 male individual of the control group. A frequent heterozygous exchange of thymine by cytosine was found at position 1284 (c.1284T>C; Tyr428Tyr), a previously known *FOXF2* sequence variation (rs2293783). It was detected in patients 2, 4, 6, 7, 10, 11, 17 as well as in 9 normal controls (4 males and 5 females). Patient 5 carried the same sequence variation in a homozygous state which was not found in any of the normal controls. The PCR fragment 6 of exon 1 contained 5 or 6 GGC repeats coding for glycine from position 904 onwards (c.904GGC(5_6);

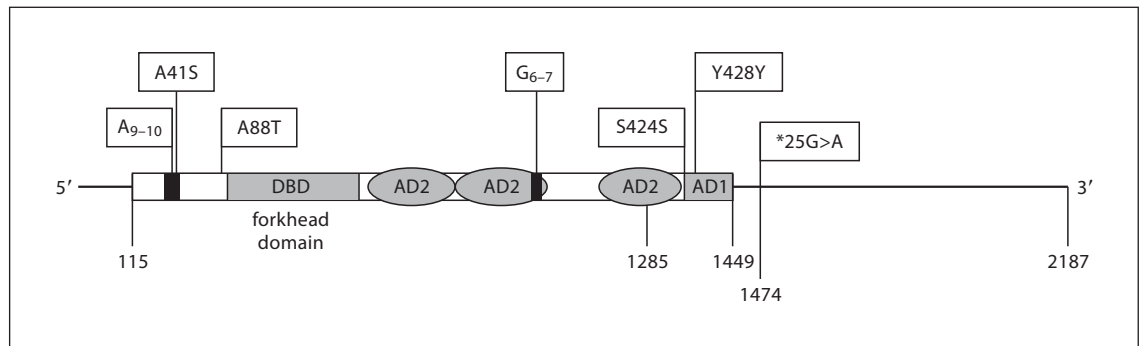


Fig. 2. Summary of the position of detected sequence variations in the *FOXF2* mRNA (NM_001452). nt.1–nt.114: 5'-untranslated region; nt.115–nt.117: Startcodon; nt.1285: end of exon 1; nt.1447–nt.1449: Stopcodon; nt.1450–nt.2187: 3'-untranslated region; nt.1474: G/A: SNP rs45600838. DBD: DNA binding domain, forkhead domain; AD1: activation domain 1; AD2: 3 regions of the

non contiguous activation function 2. Upper boxes indicate amino acid changes by SNPs within the *FOXF2* reading frame. A41S (SNP ss105106971); A_{9–10}: homopolymer stretch of 9–10 alanine residues (SNP ss105106973); A88T (SNP ss107795095); G_{6–7}: homopolymer stretch of 6–7 glycine residues (SNP rs58230522); S424S (SNP ss105106972); Y428Y (SNP rs2293783).

Table 3. Sequence variants found in the study

dbSNP accession	nt sequence variation	AA sequence variation	Patient	No. of controls
ss105106971	c.121G>T (heterozygous)	p.A41S	–	1
ss105106973	c.97GCC[9]+[10] (heterozygous)	p.A33[9]+[10]	10	–
ss107795095	c.262G>A (heterozygous)	p.A88T	3, 14	2
ss105106972	c.1272C>T (heterozygous)	p.S424S (silent)	15	1
rs2293783	c.1284T>C (heterozygous)	p.Y428Y (silent)	2, 4, 6, 7, 10, 11, 17	9
rs2293783	c.1284T>C (homozygous)	p.Y428Y (silent)	5	–
rs58230522	c.904GGC[5] (homozygous)	p.G301[6]	9, 13, 15	6
rs58230522	c.904GGC[5]+[6] (heterozygous)	p.G301[6]+[7]	1, 2, 3, 5, 6, 7, 12, 14, 16, 18	12
rs58230522	c.904GGC[6] (homozygous)	p.G301[7]	4, 8, 10, 11, 17	2
rs45600838	*25G>A (heterozygous)	3' UTR	12	–

rs58230522) in our set of individuals. The GGC repeat is preceded by a GGG codon also coding for a glycine leading to a repeat of 6 or 7 glycine residues (fig. 2). In contrast to the alanine repeat in the N-terminus, the glycine repeat is more variable. Of our 18 patients, 3 were homozygous for 5 GGC repeats, 10 were heterozygous for 5 and 6 repeats, and 5 were homozygous for 6 GGC repeats. GGC repeats were distributed similarly in the group of control individuals. Patient 12 showed a heterozygous replacement of guanine to adenine at the 3'-untranslated region (25*G>A; rs45600838) of the *FOXF2* gene. This nucleotide exchange was neither found in any of the 20 control individuals nor in any of the other 17 patients.

Discussion

Here we report for the first time on a genomic DNA sequence analysis of the *FOXF2* gene in 18 patients with DSD combined with cleft palate without a currently established endocrine or genetic diagnosis as based on the files available in our database. We identified 4 different DNA sequence alterations of the *FOXF2* gene which also occurred in the group of normal controls at a comparable frequency thus excluding their relevance for the observed phenotypes. However, 2 additional heterozygous *FOXF2* sequence variations, an expanded GCC repeat in exon 1, leading to an extra alanine (A_{9–10}) in the N-terminus of *FOXF2* and a heterozygous G>A ex-

change in the 3'-untranslated region, were present in only 1 patient each (patient 10 and 12, respectively) but neither in any of the 20 controls nor in any of the other 17 patients.

There are published biological examples in other endocrine diseases suggesting that the type of the two identified unique *FOXF2* sequence alterations in our DSD patients could play a functional role in the phenotype. In the first, a recently reported insertion of an extra CTG in the poly-leucine repeat in exon 1 of the *GNAS1* gene causing Albright's hereditary osteodystrophy (AHO) (Lim et al., 2002) has considerable similarities with the exon 1 mutation of *FOXF2* observed in our patient 10 extending the polyalanine repeat (fig. 2). Secondly, the replacement of guanine with adenine at the 3'-untranslated region (25*G>A; rs45600838) of the *FOXF2* gene in patient 12 has similarities with a previously described silent mutation within the very end of exon 8 of the androgen receptor gene causing aberrant splicing of the androgen receptor mRNA inducing partial androgen insensitivity syndrome (Hellwinkel et al., 2001). Therefore, it cannot be excluded that the 25*G>A could modify *FOXF2* mRNA transcription and thus contribute to the observed phenotype. The importance of an adequate gene dose for sexual differentiation processes has been well documented for many genes, namely *SF-1* (*NR5A1*) (Achermann et al., 2002), *WNT4* (Jordan et al., 2001), and *DAX1* (*NR0B1*) (Parma et al., 1997; Iyer and McCabe, 2004). Taken together, it may be hypothesized that the two unique *FOXF2* gene alterations as detected in patients 10 and 12 could have functional consequences on the mRNA or the protein level resulting in reduced gene function or gene dosage at critical times during genital development, thus being involved in expression of the underlying phenotypes. Unfortunately, DNA of the parents and of potential siblings was not available. Also primary tissues like cultured genital fibroblasts were not available for functional studies. Therefore, the exact role of these 2 *FOXF2* sequence alterations remains uncertain at this point.

Due to the combination of cleft palate and impaired genital tubercle outgrowth in the *Foxf2* knockout mice (fig. 1) and due to the identical expression pattern of *FOXF2* and *HOXA13* in our genome wide study (Holtérhus et al., 2003), we speculated that *FOXF2* would potentially act on the level of development of the bipotent genital anlagen but not on the level of androgen production or androgen action. Therefore, one would expect normal baseline and stimulated testosterone levels in affected patients. Unfortunately, hormonal data were

incomplete and not available in each of the 18 cases of our DNA database (table 1). In particular, there was no hormonal data available in patients 10 and 12, the ones with the unique sequence alterations. In some patients, hCG-induced rise of testosterone was only marginal, e.g., patient 2: 91 ng/dl; patient 17: 99 ng/dl. Since the latter patient carried a chromosomal mosaicism 45,X/46,XY, it is likely that this patient has gonadal dysgenesis as the underlying cause of genital undervirilization with cleft palate occurring for other currently unknown reasons.

The combination of hypospadias and cleft palate is not rare and has been described as part of different syndromes. In none of the 18 patients, a definitive diagnosis had so far been established. Patient 2 had clinical characteristics compatible with the Robinow syndrome which is characterized by dwarfism, genital hypoplasia like micropenis and reduced clitoral size, spinal abnormalities, and renal tract abnormalities. Facial features include hypertelorism with midfacial hypoplasia and a broad and prominent forehead combined with a short nose. Oral features contain a tented upper lip. A cleft palate is not a commonly described criterion (Patton and Afzal, 2002). In patient 2 only very few features are fulfilled (table 1).

Patient 12 had some clinical features related to the autosomal dominant CHARGE syndrome. Three major clinical criteria reported by Verloes (2005) are ocular coloboma, choanal atresia, and hypoplasia of semicircular canals; minor criteria are rhombencephalic dysfunction, hypothalamo-hypophyseal dysfunction, malformation of the internal or external ear, malformation of the mediastinal organs, and mental retardation. In a typical CHARGE syndrome, 3 major or 2 major and 2 minor criteria must be fulfilled (Verloes, 2005). In patient 12, none of the three majors is reported, and therefore, a typical CHARGE syndrome is excluded.

Until today, investigation of genes potentially involved in the development of the early bipotent genital anlagen outside the gonads and outside androgen-dependent sexual differentiation has been performed predominantly in mouse models (Haraguchi et al., 2000). In a more recent study, Beleza-Meireles et al. (2007) did not find mutations of clear pathogenetic relevance in the promising candidate genes *FGFR2*, *FGF8*, *FGF10*, and *BMP7* in patients with hypospadias. The latter are part of a complex developmental network of gene regulation and epithelial-mesenchymal interactions involving the urethral plate epithelium and the genital tubercle (Morgan et al., 2003). Nevertheless, it is important for the first

step towards a better understanding of normal and aberrant human genitalia development to investigate genomic mutations and variations of promising new candidate genes of early genital anlagen development in human since many DSD patients lack a well-defined molecular diagnosis yet.

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