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Screening for mutations in candidate genes for hypospadias

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Abstract Hypospadias, a condition with a frontally placed urethral orifice on the penis, is the most common malformation in males. During fetal development several components are necessary for normal male genital development. Testosterone and dihydrotestosterone act via the androgen receptor but a defective receptor function results in different degrees of genital malformations. Testosterone-5α-reductase converts testosterone to dihydrotestosterone, which is crucial for normal differentiation, and a total lack of this enzyme results, in syndromes with hypospadias. The Wilms' tumour 1 (WT1) gene is expressed in the fetal gonad and genital malformations can occur due to WT1 gene mutations. These genes are therefore strong candidate genes for hypospadias. We have analysed 35 boys with hypopadias and one girl diagnosed as with complete androgen insensitivity syndrome, using exon by exon polymerace chain reaction (PCR) amplification of the AR, WT1 and 5α-reductase genes and screened for point mutations and performed subsequent DNA sequencing. No mutations in any of these genes were found in the 26 patients with isolated hypospadias. Two patients with severe hypospadias with cryptorchidism were found to carry mutations in the androgen receptor gene. Also the girl with clinically diagnosed complete androgen insensitivity was found to be homozygous for a splice mutation in the 5α-reductase gene. In summary, mutations in the WT1, AR and 5α-reductase genes are not common causes of isolated hypospadias.

Key words Hypospadias · Wilms' tumour 1 gene · Androgen receptor gene · 5α-reductase gene · Mutations

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Introduction

Sex is determined chromosomally at conception, but for the first 6 weeks the fetus remains sexually undifferentiated. Subsequent sex differentiation and sex organ formation is completed at the fourteenth gestational week. This process involves a delicately orchestrated, well-timed interplay between several gene products including various sex hormones and their receptors, and the Wilms' tumour 1 (WT1) gene, among others [6, 15]. Any malfunction in this well-coordinated chain of events may result in an altered phenotype.

Hypospadias, an abnormally located urethral orifice along the frontal side of the penis, is the most common malformation in males, with a frequency of about 1:400. Phenotypically the malformation may vary from a slight anomaly where the urethral orifice is located on the frontal side of the tip of the penis to an extreme case with the orifice in the perineum, resulting in bifid scrotum, often combined with a curving of the penile shaft. The exact molecular mechanisms underlying this malformation remain largely unknown. The condition is thought to be polygenic and in most cases it occurs sporadically, but families exist in which hypospadias segregates as a dominant trait.

Several genes have been implicated in genital malformation syndromes, including hypospadias. Constitutional deletions of chromosome band 11p13 encompassing the WT1 gene result in the WAGR (aniridia-Wilms' tumour) syndrome (including genital malformations), whereas WT1 missense mutations in heterozygous form have been detected in patients with Drash syndrome (male pseudohermaphroditism, renal

failure and Wilms' tumour) [4, 16]. In addition nonsense mutations of WT1 have been described in a few cases with Wilms' tumour and genital malformations but without renal failure [15].

The testosterone 5α-reductase type 2 gene (SRD5A2) encodes for the enzyme that converts testosterone to dihydrotestosterone, a hormone that induces penile and scrotal development. Dihydrotestosterone and its precursor, testosterone, both act on their target organs (the prostate gland, the external genital organs and the Wolffian ducts, respectively) via binding to the androgen receptor (AR) [6]. Complete lack of 5α-reductase type 2 results in a clinical condition called pseudovaginal perineoscrotal hypospadias, characterized by a female external phenotype, normal testes and normal Wolffian structures internally [22]. A non-functioning AR results in a female phenotype in an XY infant with testes [5]. Milder dysfunctions of the AR results in phenotypes with different degrees of partial androgen insensitivity and mutations in the AR gene were found in two cases of sibling pairs with perineal hypospadias [2]. Thus the WT1, SRD5A2 and the AR genes are candidates for harbouring germline mutations in male patients with genital malformations. To explore these possibilities, we analysed the WT1, AR and SRD5A2 genes for mutations in a set of patients with hypospadias.

Materials and methods

Clinical data

Clinical diagnoses of the 36 patients analysed are listed in Table 1. All cases had a 46, XY karyotype. The patients had different degrees of severity from perineoscrotal hypospadias with bifid scrotum and curvation of the penis to penile forms. All patients had undergone reconstructive surgery. In the group with hypospadias and cryptorchidism, two were clinically diagnosed as partially androgen insensitive, one of whom had an XY sister. In addition one case of an XY female who was diagnosed as having complete androgen insensitivity was subject to this mutational screening. Among the seven families with two or more hypospadias, two families had brothers who were affected, and in three families cousins had hypospadias.

Mutational analysis with denaturing gradient gel electrophoresis (DGGE)

Genomic DNA was prepared according to standard protocols from peripheral venous blood or from fibroblast cultures when skin biopsies were taken at the operation. DNA was screened for point mutations with exon by exon specific PCR of exons 2–10 of the WT1 gene, exons 1–5 of the SRD5A2 gene and exons 2–8 of the AR gene, followed by either DGGE or single strand conformation polymorphism (SSCP) analysis (see below). The theoretical melting profiles of the individual exons were generated using the computer program Melt 87. Melting maps predicted that the coding regions of the genes analysed would all be contained in a single low melting domain. Hence, mutations occurring in those regions are likely to be detected by DGGE. The oligonucleotide primers for the different exons were designed to optimize mutation detection by DGGE by attaching a 40-bp-long GC clamp on the 5′ end of either the upstream or the downstream primer [19], according to the prediction of the melting properties of the amplified fragment. Primer sequences, PCR and DGGE conditions are listed in Nordenskjöld et al. [13] and Tables 2 and 3, respectively. The PCR was performed in a 50 µl volume containing 200 ng of template DNA, 0.2 mM dNTPs, 10–50 pmol of each primer, 50 mM KCl, 20 mM TRIS-HCl (pH 8.3), 1.0–2.5 mM $MgCl_2$, 1.25 U Taq DNA polymerase (AmpliTaq, Perkin Elmer, Branchburg, New Jersey, USA) and overlaid by 20 µl mineral oil. The thermal cycling was performed in a Perkin-Elmer Cetus Thermocycler with an initial 94°C denaturing step for 5 min, followed by 30 cycles of denaturing 94°C 1 min, a 2 min annealing step at the temperature specified in tables 2 and 3 and extension at 72°C for 1 min, followed by a final extension of 10 min at 72°C. Of the PCR product 5% was checked for successful and specific amplification on 2% agarose gels. Samples were denatured for 10 min at 94°C prior to DGGE analysis.

Parallel denaturing gradient gels were cast and run using Hoeffer Scientific Instruments, San Francisco, California, USA model SE 620, according to Myers et al. [12]. The gels contained 7% acrylamide (37.5:1 acrylamide:bisacrylamide) in a 1 × TAE buffer (40 mM TRIS acetate/1mM EDTA, pH 8.3) and were prepared from two stock solutions: 0% containing no denaturants and 100% containing 7 M of urea and 40% formamide. These solutions were mixed in appropriate amounts to generate the desired concentrations of denaturants to be used in each gel. The gels were polymerised with 1/200 volume of 10% ammonium persulfate and a 1/2000 volume of TEMED (*N*, *N*, *N*′, *N*′-tetramethylethylenediamine). The gradients were generated using a gravitational gradient mixer (Model 385, BioRad). Following polymerisation, gels were run at 85 V for 16 h, submerged in a TAE buffer heated to 62°C, with circulation of the buffer. After completed electrophoresis, gels were silver-stained and dried in a gel dryer.

Mutational analysis with single strand conformation polymorphism (SSCP)

Exons that did not amplify properly with the predicted DGGE primers or that failed to focus on DGGE gels after trying several different DGGE running conditions were screened for mutations with SSCP, as indicated in Tables 2 and 3. After end-labelling one primer with ^{35}S, PCR with intronic primers was performed and the denatured product was run on 6% polyacrylamide gels without glycerol in 4°C overnight and with 10% glycerol at room temperature overnight, and exposed to X-ray for 1–5 days.

Table 1 Clinical data

Clinical diagnosis	Number of cases	Mutation
Hypospadias isolated, penile shaft		
sporadic	13	
familial	7	
Hypospadias, penoscrotal or perineal: sporadic	6	
Hypospadias combinated with cryptorchidism	7	AR, Ser597Thr
Partial androgen insensitivity syndrome	2	AR, Phe725Val
XY female, initially diagnosed as CAIS	1	SRD5A2, splice
Total	36	

Table 2 Experimental conditions for PCR and DGGE for the SRD5A2 gene

Exon	Primers	PCR annealing temp. (°C)	PCR bf $MgCl_2$ (mM)	DGGE gel concentration (%)
1a	GCG CTC TCT TCT GGG AGG GC GCC GGC TTC AGG CTC TC	68	1.5	SSCP
1b	CGA AGC CCT CCG GCT ACG GG TGG GCG CCC GCA AGG GAA AAA CG	68	1.5	SSCP
2	AAC AGT GAA TCC TAA CCT TTC C [a]AAG GGT TGT TAG CTG GGA AGT AGG	64	2.0	20–80
3	TCC TTT CTC TCT TCT TTG AGT CTG GGG GCA GGG GAG AC	55	1.5	SSCP
4	ATT GAC CTT CCG ATT CTT CTG C [a]GAA AGC TAC GTG AAT GCT GCC	60	1.5	20–80
5	TCA GCC ACT GCT CCA TTA TAT TTAC TTC ATC AGC ATT GTG GGA GC	55	1.5	SSCP

[a] Denotes a CG-clamp: GCG GCC GCC CGT CCC GCC GCC CCC GCC CCG CCG CGG CCG C
Sequence according to Labrie et al. [9]

Table 3 Experimental conditions for PCR and DGGE for the AR gene

Exon	Primers	PCR annealing temp. (°C)	PCR bf $MgCl_2$ (mM)	DGGE gel concentration (%)
2	[a]GCC TGC AGG TTA ATG CTG AAG CC TAA AGG AGA AAG GGA AAG AG	45	2.0	20–80
3	ACT CAT TAT CAG GTC TAT CAA CTC T AAA ATC TGG TCT AAA GAG AGA CTA G	56	1.5	SSCP
4	[a]TAA ATT CAA GTC TCT CTT CC TTA TCT CAT GCT CCC ACT TCC	55	1.5	35–65
5	CAA CCC GTC AGT ACC CAG AC AGC TTC ACT GTC ACC CCA TC Nested 3′: [a] TAT TTT AGT GAG GTC TGG	45	1.5	20–80
6	TCC TTT TTC CTC TGT GTA TC [a]AAT GGC AAA AGT GGT CCT CTC	50	1.5	35–65
7	[a]CCA TTC TGT CTT CAT CCC AC ATA AAG CAC CCT CCA TCG TTT G	48	1.0	20–80
8	[a]TTG TCA ACC CTG TTT TTC TCC GAC ATC TGA AAG GGG GCA TGA G	60	1.5	20–60, 2 h short gel

[a] Denotes a CG-clamp: GCG GCC GCC CGT CCC GCC GCC CCC GCC CCG CCG CGG CCG C
Sequence according to Lubahn et al. [10]

DNA sequencing

Aberrant fragments were subject to a new PCR with one biotinylated primer and after purifying the fragment with MagicPrep (Promega), Madison, Wisconsin, USA (Promega), streptavidin-coated magnetic beads (Dynal, Norway) were used to isolate a single-stranded template. Direct sequencing was performed according to standard procedures using the Sequenase kit (US Biochemicals, Cleveland, Ohio, USA) with T7 polymerase, ^{35}S-dATP and 6% denaturing polyacrylamide gels, and exposed to X-ray for 12–24 h, without intensifying screens, at room temperature. The XY female subject was analysed for SRY gene mutations with direct DNA sequencing.

Linkage analysis in six families

The following microsatellite markers located to the vicinity of SRD5A2 gene 2p23 (D2S131, D2S149, D2S387, D2S305, D2S171), and the intragenic trinucleotide in the AR gene was used to amplify DNA for detection of linkage [20]. For the WT1 gene the CA repeat in exon 10 was analysed [7].

Results

Isolated hypospadias

In the group of boys with isolated hypospadias without associated cryptorchidism (26 cases) no evidence for mutations was found after screening in any of the three investigated candidate genes. However, it should be pointed out that mutations in the exon 1 of the AR gene are not excluded in this analysis.

WT1 gene

No mutations were found. The expected allele frequencies for polymorphisms in exon 3, 7 and 8 were found (0.3, 0.13 and 0.1 respectively for the rare alleles).

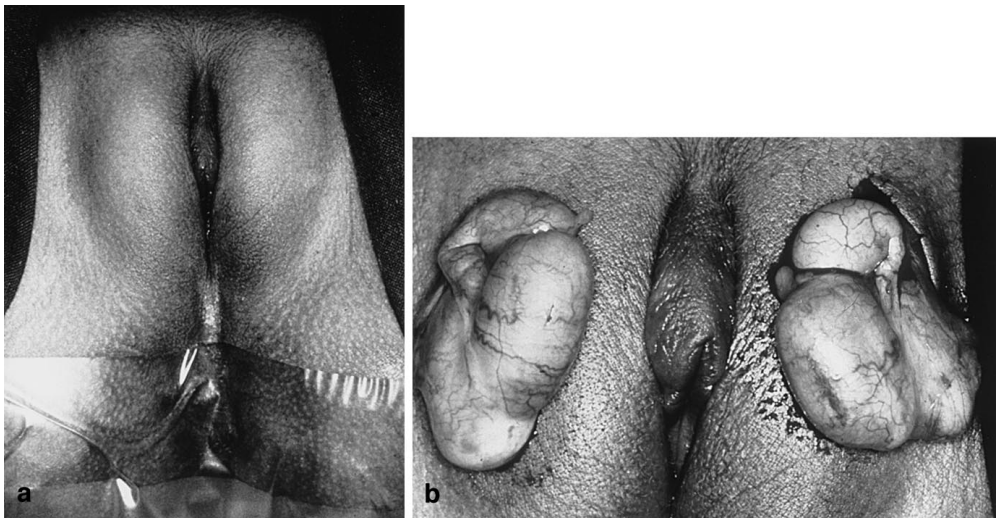


Fig. 1a, b Photographs taken just before and during surgery, displaying normal testes and epididymis

The 5α-reductase gene

The single case of an XY female (case 28) was additionally investigated in this study. Previously diagnosed as having complete androgen insensitivity, she was a phenotypic girl born with a slight clitorimegaly and palpable gonads in the pubic region. The karyotype was 46, XY. Biopsies taken from the gonads showed a normal testicular histology. At an explorative laparotomy no ovaries or uterus were found. Photographs taken just before and during surgery are shown (Fig. 1). She was assigned as a female and the gonads were removed. The girl also had a female relative who was masculinised. The DGGE analysis of the WT1 gene and the AR gene showed no abnormalities. However exon 4 of the SRD5A2 gene revealed a homozygous abnormal band (Fig. 2a). DNA sequencing showed a homozygous point mutation involving the first intronic base pair after exon four (g→t) (Fig. 2b). This mutation has previously been described in a Pakistani ethnic group with 5α-reductase gene type 2 deficiency [5]. This girl was also of Pakistani origin. No other abnormalities in this SRD5A2 gene were detected in this study after screening with DGGE (exons 2 and 4) and with SSCP (exons 1, 3 and 5).

The androgen receptor gene

In one subject (case 19) with hypospadias, and cryptorchidism clinically diagnosed with partial androgen insensitivity based on sparse body hair, gynaecomastia and heredity for intersex malformations, an abnormality in exon 5 was detected with DGGE (not shown). Subsequent DNA sequencing showed a C to T substitution in codon 725, changing Phe to Val (Fig. 3). One other boy (case 9) with severe hypospadias, bifid scrotum and cryptorchidism exhibited an abnormal band on SSCP for exon 3 of the AR gene (Fig. 4a) and subsequent DNA sequencing showed a G to C transition in codon 597, changing Ser to Thr (Fig. 4b). This mutation changes the second codon in the sequence coding the dimerization signal.

In addition, a silent polymorphism was found in exon 7 of the AR gene in a boy (case 4) with hypospadias and

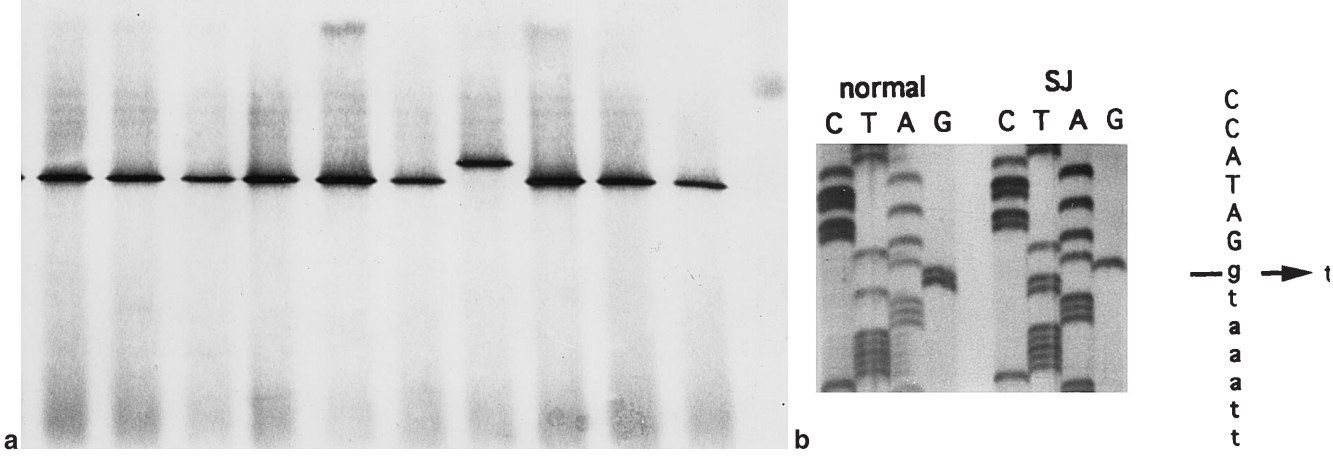


Fig. 2 a Denaturing gradient gel electrophoresis (DGGE) of the 5α-reductase type 2 gene exon 4 showing the homozygous abnormal band in case 28, situated above the normal bands surrounding it. **b** DNA sequence analysis of case 28 (*SJ*) showing a change of the first intronic base pair after exon 4 (g → t) thereby changing the splicing signal

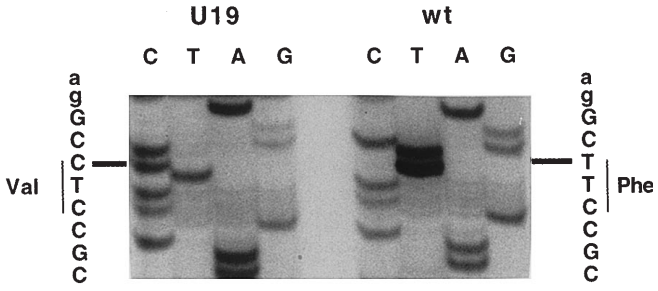


Fig. 3 DNA sequence analysis showing a T → C mutation in the androgen receptor gene exon 5 in case 19 Phe 725 → Val

cryptorchidism changing CTC (Leu) to CTT (Leu) in codon 838 (Fig. 5a, b).

Linkage analysis

In six of the seven families, linkage was excluded for all three genes, since affected males did not share alleles for the polymorphisms studied, or the markers were not informative (data not shown).

Discussion

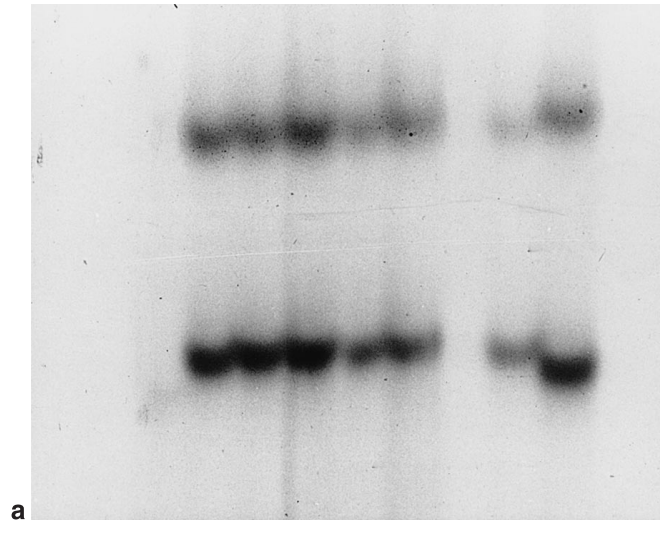
In this study, three candidate genes implicated in male genital malformations were analysed in a heterogenous group of patients with such disorders. Strikingly, no mutations within any of the analysed genes were demonstrated in patients with isolated hypospadias. Despite the fact that patients with hypospadias are at an increased risk for Wilms' tumour development [11, 17, 21], only a few germline mutations within the WT1 gene have been reported in patients with hypospadias, and in those cases only in combination with Wilms' tumour. Moreover, XY females with gonadal dysgenesis as well as males with severe hypospadias or micropenis without clinical signs of androgen insensitivity did not display any WT1 mutations [14, 3]. Thus, it seems that mutations within the WT1 gene, rarely occur in genitourinary malformation without Wilms tumour. Therefore the strongest phenotype of WT1 gene mutations is indeed the tumour. This notion is now further supported by this study, where WT1 gene mutations were not found in cases with isolated genital malformations without an associated cancer.

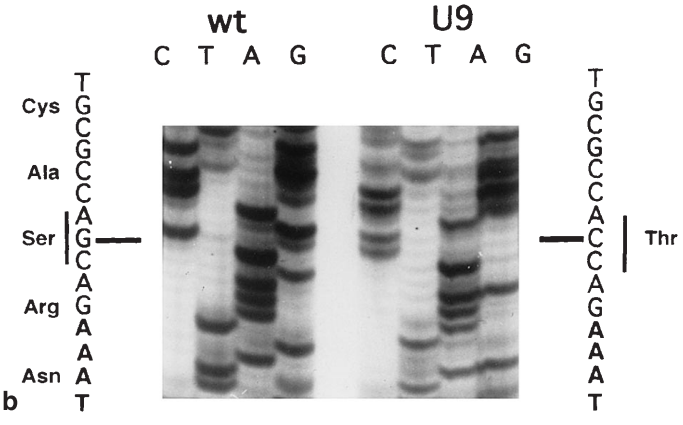
Mutations within the SRD5A2 gene were not found in any patient with isolated hypospadias. Taken together with the fact that male carriers for such mutations do not show genital anomalies, it indicates that mutations within this gene rarely cause isolated hypospadias. However, it is possible that selected material from adult males with hypospadias in association with small prostate glands could display the mildest phenotype of a SRD5A2 gene mutation.

AR gene mutations also seems to be an uncommon cause of isolated hypospadias if additional signs of androgen insensitivity or heredity are absent. Recently, similar conclusions were drawn from two studies of 9 and 21 patients, respectively. Only one case of disease causing mutation in each of those studies was detected in patients with severe hypospadias [1, 8].

Of the two cases that were diagnosed as partially androgen insensitive, one showed an exon 5 missense mutation in the AR gene and the other has so far no known mutation, although exon 1 was not analysed. This latter case had micropenis, hypospadias, bifid scrotum, but no other signs of androgen insensitivity, and no known heredity. One additions boy with penile hypospadias combinated with cryptorchidism and bifid scrotum had a mutation in exon 3 of the AR gene. No additional mutation was found in the present study. The XY female, who was originally diagnosed as having

Fig. 4 a Single strand conformation polymorphism (SSCP) of the androgen receptor gene exon 3 showing an abnormal band in case 9, loaded to the right. **b** DNA sequence analysis of case 9 showing a change in codon 597 from G → C, which replaces Ser with Thr





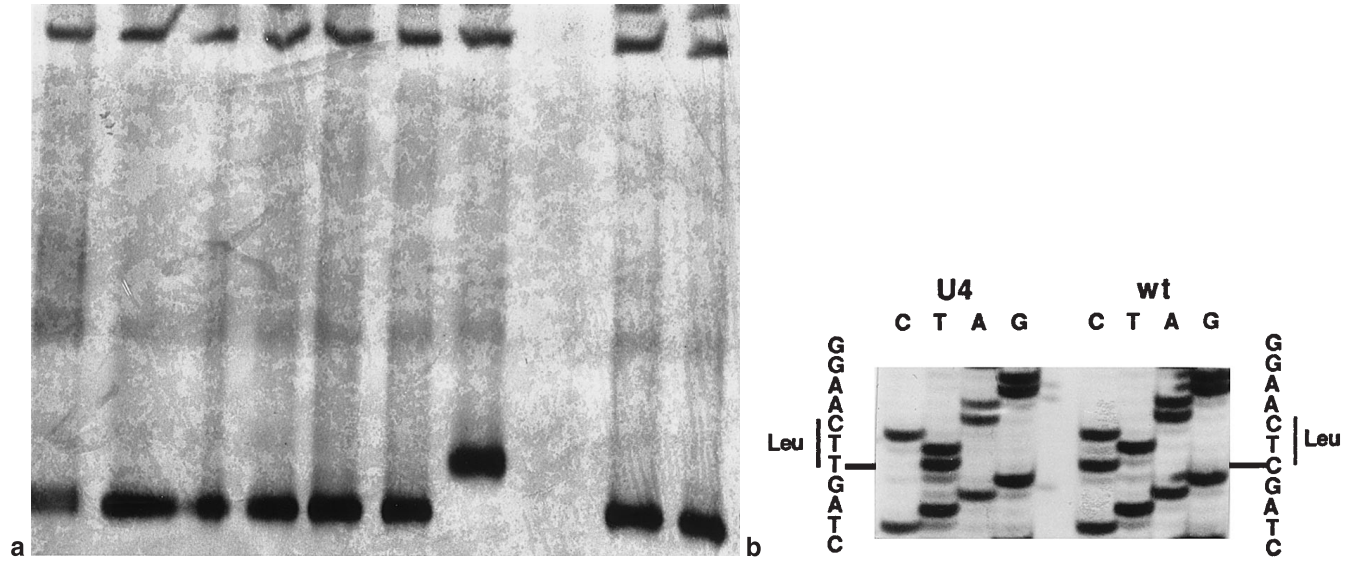


Fig. 5 a DGGE of the androgen receptor gene exon 7 showing an abnormally placed band for a patient (case 4) with hypospadias and cryptorchidism. **b** Sequence analysis showing a change in base pair from CTC (Leu) to CTT (Leu) in codon 838, not found in any of the other patients

complete androgen insensitivity while the correct diagnosis is 5α-reductase type 2 deficiency, illustrates that the molecular and clinical diagnoses are not always congruent. In the future, the molecular background of a disease is likely to be more integrated into clinical diagnostics. In the case of 5α-reductase type 2 deficiency the decision on the child's sex must be considered carefully, since the child will undergo a natural virilisation during puberty and could be fertile if the male sex is chosen.

The low mutation frequency in three strong candidate genes in a selected population with genital anomalies may stem from several reasons. First is incomplete mutational analysis, since it should be pointed out that the analysis performed in the present study did not exclude the possibility that a low fraction of mutations remained undetected. No mutational screening method will detect all mutations, especially when SSCP screening is used. In addition, due to technical reasons, exons 1 of the AR and the WT1 genes were not studied. However, the ability to detect known polymorphisms and the few novel mutations, argue strongly that the majority of the genes were adequately screened. Another explanation is that there could be expression anomalies of a gene; a possibility that is not ruled out in this study. Further insight into the molecular mechanisms of hypospadias may be gained from analysis of families where hypospadias segregates in an apparent autosomal dominant manner.

Yet another possibility for explaining the paucity of genetic alterations is that milder and sporadic forms of hypospadias result from environmental factors. Recently it has been suggested that the increasing incidence of hypospadias is the result of higher amounts of oestrogenic chemicals in the environment [18]. In summary, mutations in the AR gene, the SRD5A2 gene or the WT1 gene are probably rare causes of isolated hypospadias.

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