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## Molecular features and clinical phenotypes in androgen insensitivity syndrome in the absence and presence of androgen receptor gene mutations

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**Abstract** Androgen insensitivity syndrome (AIS) is characterized by deficient or absent virilization in 46,XY individuals despite normal or even elevated androgen levels. AIS is usually caused by mutations in the androgen receptor (AR) gene. We aimed at contrasting clinical, biochemical, and molecular genetic characteristics of three patients (P1–P3) with clinically evident partial (P1) and complete (P2, P3) AIS with and without AR gene mutations. AR expression was studied in cultured genital skin fibroblasts (GSF) by Western immunoblotting, ligand binding analyses, Northern blotting, semiquantitative reverse transcription–polymerase chain reaction (RT-PCR), and RT-PCR spanning exons 1–8. AR gene DNA sequence was analyzed by single-strand conformation analysis

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(SSCA), and DNA sequencing. GSF revealed reduced (P1) or absent (P2, P3) ligand binding. Northern blots showed either slightly reduced hybridization of the 10.5-kb AR transcript (P3) or no hybridization (P1, P2), as confirmed by semiquantitative RT-PCR. RT-PCR spanning exons 1–8 detected single AR mRNA bands in P1–P3 excluding splicing errors. Western analyses showed either low (P1) or no (P2, P3) AR protein. While SSCA initially did

not reveal any molecular abnormality, sequencing showed a novel CAG (Gln) to TAG (stop) mutation at codon 59 (P3) and a previously described 2-bp deletion at codon 472, leading to a frameshift and premature stop in codon 499 (P2). Intriguingly, P1 showed an unaltered DNA sequence of the coding region of the AR gene including all intron–exon boundaries. In conclusion, patients with clinically evident complete AIS are likely to harbor an AR gene mutation, demanding that the two polymorphic regions must always be included in molecular analyses of the AR gene. Moreover, our data support the concept that in a subset of AIS patients, particularly those with partial AIS, molecular alterations outside the coding region of the AR gene must be presumed.

**Keywords** Androgen insensitivity syndrome · Male pseudohermaphroditism · Androgen receptor · Mutation

## Introduction

Normal male sexual differentiation in 46,XY individuals is dependent on normal action of androgens via the androgen receptor (AR) in the androgen-responsive genital target tissues [1, 2]. In androgen insensitivity syndrome (AIS), normal virilization of the genital structures does not take place because of an end-organ resistance to androgens. Clinically, AIS is characterized by a wide spectrum of genital phenotypes. It ranges from complete AIS with normal female external genitalia over different forms of partial AIS with genital ambiguity to mild forms (minimal AIS) characterized by only slightly diminished virilization or isolated infertility but phenotypically normal male external genitalia [3, 4].

On the molecular level, AIS is usually caused by X-chromosomal recessively inherited mutations of the AR gene, leading to functional impairment of the AR with respect to its core task as a ligand-activated transcription factor of androgen-regulated target genes. Functional deficiency has been reported to be due to the expression of a functionally defective AR protein [5, 6] or by quantitatively reduced or absent AR protein expression due to gene deletions [7, 8], premature translation termination codons [9], and splice site mutations [10–12] and modified translation initiation consensus sequences [13]. In addition to their functional impact, AR gene mutations may also lead to a reduction of AR mRNA [5] or AR protein in genital target tissues [14]. There is currently only one published case in which the authors suggested AIS to be caused by isolated deficiency in the level of coregulators of the AR signaling pathway, supporting the existence of possible factors outside the AR gene leading to AIS [15]. Based on these considerations, molecular analysis of the AR gene is the gold standard in verifying the diagnosis of AIS. However, in a subset of patients with genital malformations presumed to have AIS (i.e., male pseudohermaphroditism with normal T secretion in which 5 $\alpha$ -reductase deficiency has been ruled out), no molecular abnormality within the AR gene can be detected.

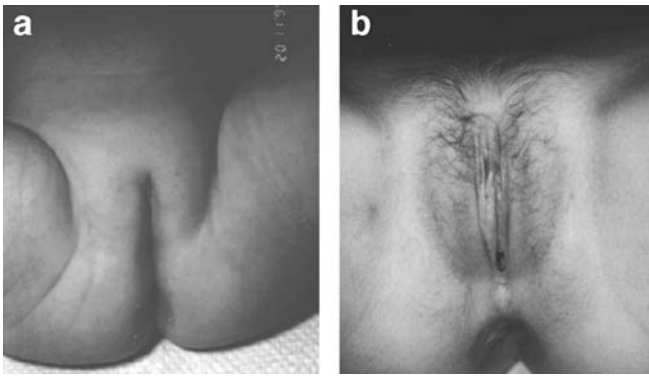
Here we present three unrelated 46,XY individuals in whom clinical and biochemical data strongly suggested the diagnosis of AIS. AR expression studies using cultured genital skin fibroblasts (GSF) clearly confirmed the diagnosis of AIS on the mRNA level, the protein level, or both in all three patients. While single-strand conformation analysis (SSCA), a highly sensitive method to detect sequence variation within conserved DNA amplicates, did not reveal any abnormalities, DNA sequencing of the whole coding region of the AR gene uncovered the existence of disruptive mutations near or within the variable repeat regions within the first exon of the AR gene in two patients. In contrast, in one patient, neither SSCA nor DNA sequencing revealed an underlying mutation. Our data indicate that careful DNA sequencing of the variable trinucleotide repeat regions within the first exon should be included in molecular analysis of the AR gene if no mutation is detected with other methods. However, a subset of patients with AIS does not carry mutations within the coding region of the AR gene despite major reduction of AR protein expression.

## Materials and methods

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

**Patient 1 (P1)** P1 presented at birth with ambiguous external genitalia (AIS type 3 according to Sinnecker's classification) [16]. The family history was uninformative in this as well as in the other two patients. Chromosome analysis revealed a normal 46,XY karyotype. At the age of 2 months, P1's serum testosterone (T) level was 9.43 nmol/l (normal male range 3.1–8.8 nmol/l) and serum dihydrotestosterone (DHT) level was 3.23 nmol/l (normal male range 0.2–4.5 nmol/l), demonstrating a normal T/DHT ratio (3.25), thus ruling out a defect of testosterone biosynthesis as well as 5 $\alpha$ -reductase type II deficiency. Molecular analysis of genomic DNA of the patient could not prove mutations of the *SRD5A2* gene. Because of presumed AIS, a sex hormone binding globulin (SHBG) androgen sensitivity test in response to 0.2 mg kg<sup>-1</sup> day<sup>-1</sup> of the anabolic substance stanozolol taken orally on three consecutive days was performed. This test showed SHBG suppression from 122.6 nmol/l before administration of stanozolol to 87.2 nmol/l at day 7, corresponding to 71.1% of the initial value (normal response <63.4% [16]), indicative of a partial defect of in vivo AR function. When P1 was 8 months of age, his gonads were removed. The right gonad was located in the inguinal region, whereas the left gonad was descended into the scrotum. Histological examination of the gonads proved the presence of normal testicular tissue with focal Leydig cell hyperplasia, supporting the clinical and biochemical diagnosis of AIS.

**Patient 2 (P2)** P2 presented with normal female external genitalia at the age of 1 year (Fig. 1a). Diagnosis was initiated at this time because the right gonad was palpable



**Fig. 1** External genital phenotypes of P2 (a) and P3 (b)

in the inguinal region. Ultrasound examination showed that no uterus was present. Genitography showed a short and blind-ending vagina. Chromosome analysis revealed a normal 46,XY karyotype. Basal T level was 1.38 nmol/l (normal male range 0.1–0.5 nmol/l) and DHT level was 0.31 nmol/l (normal male range 0.05–0.2 nmol/l) in serum. Following administration of a single dose of 5,000 U hCG, T and DHT levels increased to 10.32 and 1.0 nmol/l, respectively, excluding a relevant defect of T and DHT biosynthesis. Although the gonads were not biopsied, a genital skin sample was taken, and fibroblasts were cultured for further studies.

**Patient 3 (P3)** The family history was inconspicuous. At the age of 4 years, the patient was operated on for an inguinal hernia on the right side; further diagnostic tests were not done. At the age of 14.6 years, P3 presented again because of vaginal infection and the absence of menarche. The external genitalia were those of a normal female. While breast development corresponded to Tanner stage 4, pubic hair was only Tanner stage PH 2 (Fig. 1b), and axillary hair was not present. Ultrasound and magnetic resonance imaging failed to detect a uterus. Vaginocopy demonstrated a short (2 cm) and blind-ending vagina. A normal 46,XY karyotype was found in blood lymphocytes as well as in GSF. Serum luteinizing hormone (LH) level was elevated to 36.3 U/l (normal male range 1.5–4.9 IU/l). Serum T was 14.87 nmol/l (normal male range 1.0–16.3 nmol/l), confirming the clinical diagnosis of complete AIS. In the SHBG suppression test, SHBG rose from 43 to 51 nmol/l, corresponding to 118% of the initial value, indicating complete absence of *in vivo* AR function. When the gonads were removed, histological examination confirmed the presence of testicular tissue, showing also focal Leydig cell hyperplasia.

#### Cell strains and cell culture conditions

Genital skin fibroblasts of all three patients (P1–P3) were obtained by biopsy from the labia majora for diagnostic purposes. Two different male GSF cultures (nGSF33, nGSF62) were derived from foreskin specimens. Cells were cultured at 37°C with 5% CO<sub>2</sub> in Dulbecco's

modified Eagle's medium with the nutrient mix F12 (Life Technologies, Grand Island, NY, USA), supplemented with 10% (v/v) fetal calf serum, 1% (v/v) MEM nonessential amino acids (Life Technologies), and penicillin (200 IU/ml)/streptomycin (0.2 mg/ml). For relative quantification of AR transcript levels, three additional strains of normal male scrotal fibroblasts were cultured under the same conditions. The identity of all cell strains (biopsy localization) has been confirmed independently from the medical records by cDNA microarray expression profiling ([2] and unpublished data). For functional analyses, cells in fourth to eighth passage were used. To enable comparison of AR expression studies, cells were always cultured at confluency according to a previously published protocol [2] with minor modifications.

#### RNA extraction

Total RNAs of GSF cell cultures were extracted using Trizol (Invitrogen) according to the instructions of the manufacturer.

#### Northern blot analysis

Northern blot analysis was performed according to Sambrook and Russel [17]. In brief, for Northern analysis of AR mRNA expression, equal amounts (20 µg) of total RNA per lane confirmed by ethidium bromide staining were separated on a denaturing formaldehyde–agarose gel containing 2% formaldehyde and 1.25% (w/v) agarose. After electrophoresis, the RNA was partially hydrolyzed by incubating the gel in 5 gel volumes of 50 mM NaOH for 20 min. Then the gel was neutralized by incubation for 40 min in 10 gel volumes of 20× standard saline citrate (SSC; 3 M sodium chloride, 300 mM sodium citrate, pH 7) and without delay blotted onto Hybond NX membrane (Amersham) by capillary action. The RNA was fixed onto the membrane by UV cross-linking (0.120 J) and hybridized. A digoxigenin (Dig)-labeled AR-specific probe was generated by incorporation of DIG-dUTP (Roche, Germany) by polymerase chain reaction (PCR) using the same primer pair and conditions as described for the reverse transcription–PCR (RT-PCR). As template DNA, a cloned AR cDNA (pSVAR0, a generous gift from Dr. Albert Brinkmann, Rotterdam, the Netherlands) was used [18]. Nonincorporated labeled nucleotides were removed from the probe using the High Pure PCR Product Purification Kit (Roche). The membrane was blocked at 50°C in standard hybridization buffer [2% blocking reagent (Roche), diluted from a 10% stock in 100 mM maleic acid, 150 mM NaCl (pH 7.5) 5× SSC, 50% deionized formamide, 0.1% *N*-laurylsarcosine, 0.02% sodium dodecyl sulfate (SDS)] for 1 h. Subsequently, the denatured probe was added (50 ng/ml) and hybridized for 16 h at 50°C. After hybridization, the blot was washed twice with 2× SSC, 0.1% SDS for 5 min at room temperature and twice with 0.1% SSC, 0.1% SDS for 15 min at 68°C. Detection of the blot was performed using

anti-DIG-AP Fab fragments and CDP-Star according to the instructions provided by the manufacturer (Roche).

#### Exon 1–8 RT-PCR

Exon 1–8 RT-PCR was carried out to qualitatively exclude potential AR mRNA splicing errors. RT-PCR was carried out between primers complementary to exon 1 (hARe1s, sense primer: 5'-TGG ATG GAT AGC TAC TCC GG-3') and exon 8 (hARe8a, antisense primer: 5'-GAG GAG TAG TGC AGA GTT ATA A-3') of the human AR. For cDNA synthesis, 2 µg of total RNA, 2 pmol antisense primer hARe8a, and 1 µl of deoxyribonucleotide triphosphates (dNTPs) (10 mM each) in a total volume of 13 µl nuclease-free water were heated to 65°C for 5 min and then incubated on ice for at least 1 min. Subsequently, 4 µl of 5× first-strand buffer (Invitrogen), 1 µl dithiothreitol (100 mM), 1 µl of recombinant RNase inhibitor (RNasin, 40 U/µl, Promega), and 1 µl of SuperScript III Reverse Transcriptase (200 U/µl, Invitrogen) were added and incubated at 55°C for 1 h. Afterwards, the reaction mixture was inactivated by heating to 70°C for 15 min. For PCR analysis, 2 µl of the RT reaction was amplified in a 20-µl reaction containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.001% (w/v) gelatin (pH 8.3), 200 µM dNTPs, 4 pmol of each of the primers hARe1s and hARe8a, and 0.5 U AmpliTaq DNA Polymerase (Roche). The AR-specific fragment was amplified in a Mastercycler gradient (Eppendorf, Germany) using the following cycling scheme: denaturation, 94°C for 15 s; primer annealing, 60°C for 30 s; chain elongation, 72°C for 2 min for 40 cycles. The amplification products were analyzed on 1% agarose/Tris-borate-EDTA gels and visualized by staining with ethidium bromide.

#### Relative quantification of AR transcript levels

One microgram of total RNA was reverse-transcribed into cDNA in a 20-µl reaction volume using 3 µg random hexamers (Roche), 2 µl 10 mM dNTPs, 2 µl 0.1 M dithiothreitol (DTT), 4 µl 5× buffer, 10 U Protector RNase Inhibitor (Roche), and 50 U SuperScript II RNase H<sup>-</sup> Reverse Transcriptase (Invitrogen). The reaction was incubated for 60 min at 37°C. The housekeeping gene porphobilinogen deaminase (*PBGD*) was amplified using the primers 5'-AGA-GTG-ATT-CGC-GTG-GGT-ACC-3' (sense) and 5'-GGC-TCC-GAT-GGT-GAA-GCC-3' (antisense). The AR cDNA was amplified using primers spanning exons 1–3 [5'-TGG-ATG-GAT-AGC-TAC-TCC-GG-3' (sense), 5'-CGA-AGA-CGA-CAA-GAT-GGA-CAA-3' (antisense)]. For relative quantification, real-time fluorescence RT-PCR was performed using the LightCycler (Roche) with the LightCycler FastStart DNA Master SYBR Green I Kit (Roche) according to the manufacturer's instructions. Each reaction mix contained 2 µl of the cDNA reaction, 2 µl of the FastStart DNA Master SYBR Green I reaction mix, 4 mM MgCl<sub>2</sub>, and 2 µl of each primer, adjusted to a total

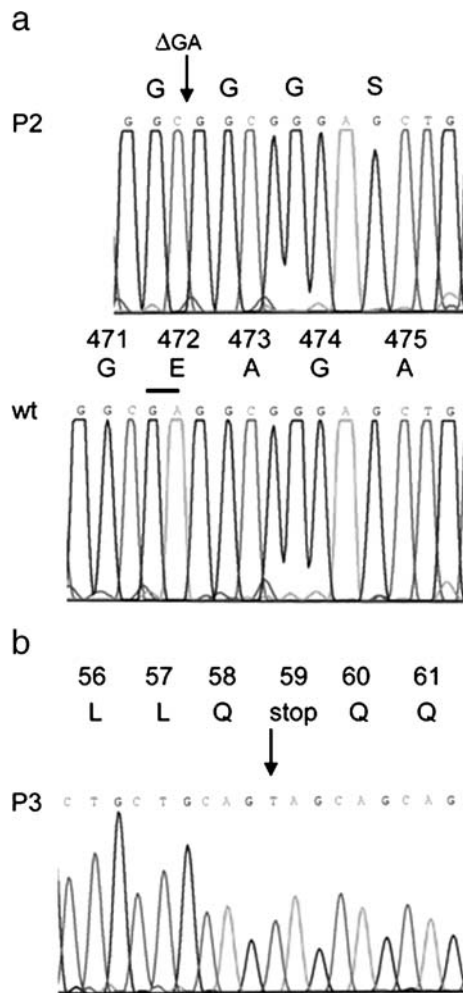
volume of 20 µl with H<sub>2</sub>O. Final primer concentrations were 2 µM per primer for *PBGD* and 1 µM per primer for the AR, respectively. The amplification scheme of the AR started with an initial denaturation of 10 min at 95°C. This was followed by denaturation (95°C for 10 s), primer annealing (63°C for 10 s), and chain elongation (72°C for 16 s and 85°C for 3 s for a total of 45 cycles). Relative quantification of AR transcript levels was calculated at the threshold cycle when the reaction just reached the exponential phase of amplification. Transcript levels were expressed as the relative ratio of the AR transcript and the *PBGD* reference measured from the same sample in the same PCR run. The mean of three experiments was determined. Calculations were performed using the LightCycler Relative Quantification Software (version 1.01, Roche). All PCR products were additionally visualized by 2% agarose gel electrophoresis (data not shown).

#### Western blot analysis

Western immunoblot analyses were performed as described elsewhere [9]. In brief, confluent cultures of labial skin fibroblasts (175 cm<sup>2</sup>) were harvested using lysis buffer with 40 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10% glycerol, 10 mM DTT, 1% Triton X-100, 0.08% SDS, 0.5% sodium deoxycholate, 600 µM phenylmethylsulfonyl fluoride, and 500 µM bacitracin. Protein samples were separated by SDS polyacrylamide gel electrophoresis for 60 min at 200 V and subsequently electroblotted on cellulose nitrate membranes for 1 h at 100 V. One hundred four micrograms of protein was loaded on each lane to compare signal intensities. The monoclonal anti-AR antibody F34.9.1 (InnoGenex, Inc., San Ramon, CA, USA) was used for AR protein detection on the dried, rinsed, and blocked membranes. An antirabbit peroxidase conjugate (Sigma) was used as secondary antibody in a 1:4,000 dilution. Protein detection was performed by chemoluminescence using the Renaissance Western Blot Chemoluminescence Reagent (DuPont NEN, Boston, MA, USA). Equal protein load of single lanes in Western analyses was confirmed further by the additional immunodetection of the constitutively expressed actin.

#### Ligand binding analyses

Androgen binding was studied using cultured GSF of P1–P3 as well as the two normal strains nGSF33 and nGSF62 as described earlier [9]. Cells were cultured to confluence and then incubated in duplicate using culture medium containing 0.02, 0.05, 0.1, 0.3, 1.0, and 3.0 nM of 17β-hydroxy-17α-[<sup>3</sup>H]-methyl-4,9,11-estrotrien-3-one ([<sup>3</sup>H]R1881) (DuPont NEN) in either the presence or absence of a 200-fold molar excess of unlabeled hormone. Scatchard calculations ( $K_d$ ,  $B_{max}$ ) were performed with Microsoft Excel Personal Computer software (Microsoft Corp., Richmond, VA, USA).



**Fig. 2** Partial AR DNA sequences of P2 (a) and P3 (b). Arrows indicate the molecular aberration compared with the wild-type sequence

### DNA studies

Genomic DNA was extracted from peripheral blood leukocytes. All eight exons of the AR gene of the patients were individually amplified by PCR including the adjacent intron–exon boundaries as described earlier [19, 20]. While exons 2–8 were separately amplified and each amplification product extended over a complete single exon, exon 1 was separated into seven overlapping segments. Subsequently, screening for mutations was performed using SSCA. In addition, the whole AR gene of each of the three patients was sequenced according to previously published protocols (Fig. 2) [19, 20].

## Results

### Northern blot analysis

Northern blot analysis showed the expected hybridization signal demonstrating a 10.5-kb AR-specific transcript using total RNA of the two normal control fibroblast cell

strains nGSF33 and nGSF62 (Fig. 3, lanes 1 and 5, respectively). Compared with these samples, P3 showed a slightly reduced signal intensity (Fig. 3, lane 4). In contrast, only very faint signals just slightly above the detection level were visible in P1 and P2 (Fig. 3, lanes 2 and 3, respectively).

### Exon 1–8 RT-PCR

RT-PCR using primers spanning exons 1–8 showed clear signals at the expected length in the normal controls (Fig. 3, lanes 1 and 5) as well as in all three patients (Fig. 3, lanes 2–4) with no extra bands (data not shown), supporting the absence of potential intronic splice site mutations.

### Relative quantification of AR transcript levels

AR/PGBD ratios were strikingly higher in the two normal male fibroblast samples (nGSF33=12.14 and nGSF62=24.47) compared with P1 (0.81), thus demonstrating a 15- to 30-fold decreased transcription in P1. In P2, the ratio was 0.24, indicating a 50- to 100-fold lower transcription in P2 compared with the control samples. P3 revealed a ratio of 13.43, which is in the range of the analyzed control samples. In addition, AR/PGBD ratios were determined within three normal male scrotal fibroblasts. In these cells, ratios were also clearly higher (6.84, 4.41, and 13.84) compared with fibroblasts of P1 and P2 (Fig. 3).

### Western blot analysis

Western immunoblot analyses revealed the expected AR-specific signal at 110–112 kDa in the two normal GSF strains nGSF33 and nGSF62 (Fig. 3, lanes 1 and 5). While P2 and P3 showed no AR-specific signal (Fig. 3, lanes 3 and 4, respectively), P1 demonstrated a very weak band at 110–112 kDa (Fig. 3, lane 2).

### Ligand binding analyses

The control cell lines nGSF33 and nGSF62 showed normal specific androgen binding (Fig. 3, columns 1 and 5, respectively; Fig. 4). In P1, severely reduced maximum androgen binding in the presence of a normal dissociation constant was detected (Fig. 3, column 2; Fig. 4). In P2 and P3, no specific androgen binding could be detected (Fig. 3, columns 3 and 4; Fig. 4).

### DNA studies

All eight exons of the AR gene including the intron–exon boundaries could be amplified successfully from peripheral blood leukocyte genomic DNA in all three patients and in the normal control subjects. However, SSCA did not reveal

**Fig. 3** AR expression data table. On the *leftmost* column, the type of experiment is listed. The hybridization signal in the *Northern blot* lane corresponds to the 10.5-kb AR fragment. The *qualitative RT-PCR* extends over exons 1–8 and is an end-point determination. The AR-specific signal in the *Western blot* lane corresponds to a molecular mass of 110–112 kDa. Equal protein load of single lanes in Western analysis has been confirmed by the additional immunodetection of the constitutively expressed actin. *Top*, represents the lane/data column as well as the used GSF strain. The normal male range of androgen binding data in cultured GSF is  $K_d=0.03$ – $0.13$  nM and  $B_{max}=13.35$ – $115.98$  fmol/mg protein

Lane / column	1	2	3	4	5
Subject	nGSF33	P1	P2	P3	nGSF62
Northern blot					
Qualitative RT-PCR (exons1-8)					
Semi-quantitative RT-PCR (exons1-3) AR/PBGD ratio	12.14	0.81	0.24	13.43	24.47
Western blot	Anti-actin				
	Anti-AR				
Ligand binding					
$K_d$ (nM)	0.08	0.05	0	0	0.08
$B_{max}$ (fmol/mg prot.)	26.4	6.43	0	0	86.16

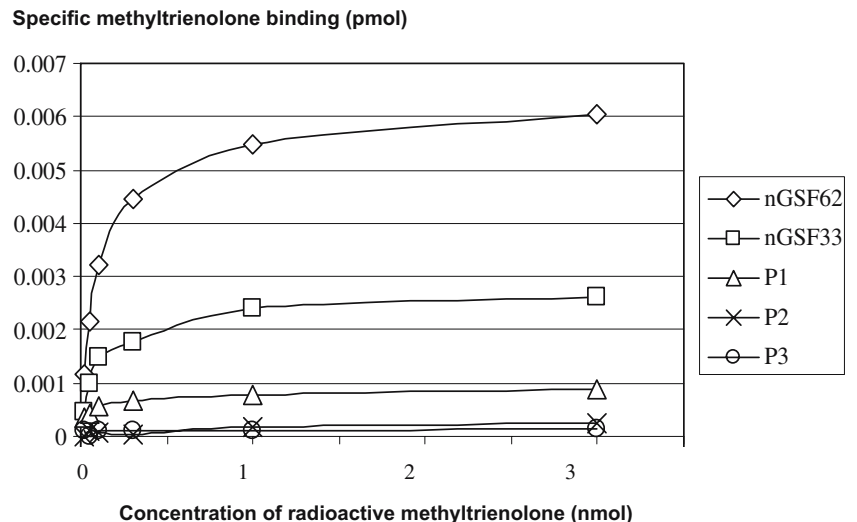
abnormalities in any of the three patients. In contrast, additional DNA sequencing of the amplified DNA fragments revealed specific alterations of the AR gene DNA sequence in P2 and P3 (Fig. 2a,b).

In P2, a 2-bp deletion at the 3' end of the polymorphic GGN repeat at codon position 472 according to the sequence published by Lubahn et al. [21] was detected (Fig. 2a). This mutation predicts for a frameshift in the open reading frame resulting in a premature termination of translation at codon position 499. The same mutation has been reported previously by Thiele et al. [22]. The mutation must have occurred independently in these two

individuals since the polymorphic repeats differed remarkably between our patient and the patient reported by Thiele et al. [22]. P2 had a polyQ tract of 21 and a polyG tract of 28 residues, while the patient reported by Thiele et al. exerted a longer polyQ tract of 24 and a shorter polyG tract of 22 residues.

In P3, we detected a CAG (Gln) to TAG (stop) mutation at codon position 59 at the 5' end of the polymorphic CAG repeat, predicting for the direct introduction of a premature termination of translation codon. While in P3 the mutation occurs in the second CAG of the polyglutamine repeat corresponding to codon position 59 (Fig. 2b), a similar but

**Fig. 4** Saturation curves of methyltrienolone binding in cultured GSF obtained from P1, P2, and P3 as well as of the normal foreskin fibroblast strains nGSF33 and nGSF62. Binding data deduced from Scatchard calculations are summarized in the AR expression data table (Fig. 2)



not identical constellation has been reported by Zoppi et al. [23] attributing the same mutation to codon position 60.

In contrast to P2 and P3, DNA sequencing in both the sense and antisense directions revealed no mutation in P1. In particular, there was no indication for a somatic mosaicism of an AR mutation in P1.

## Discussion

P3 showed the typical clinical pattern of a patient with complete AIS. This is characterized by completely female external genitalia, normal breast development, and scanty axillary and pubic hair in association with a 46,XY karyotype. This was confirmed by a characteristic constellation of biochemical data showing elevated serum concentrations of LH and T at puberty and a completely absent decrease of SHBG in response to oral stanozolol, indicating complete *in vivo* resistance to androgen. In contrast, diagnosis of AIS is more complicated in early childhood due to quiescence of the hypothalamic–pituitary axis as demonstrated in P1 and P2. Interestingly, the SHBG response to stanozolol in P1 strongly suggested a partial *in vivo* resistance to androgen corresponding well with the ambiguous external genital phenotype. Histological examination of the gonads clearly supported the clinical diagnosis of AIS in P1 and P3 as focal Leydig cell hyperplasia was demonstrated.

To further substantiate the clinical suspicion of AIS as the underlying cause of the virilization deficit, we performed AR expression studies in cultured GSF of all three patients. Western immunoblot analyses supported the diagnosis of AIS in all three patients by showing markedly reduced expression of an AR protein with normal molecular weight in P1 and completely absent expression in P2 and P3. These data were confirmed independently by ligand binding studies demonstrating significantly reduced maximum binding in P1 and negative binding in P2 and P3.

To gain further insight into the cause of AIS and the cause of reduced AR protein expression in the presented patients, we extended our study on the AR mRNA level. Using primers spanning exons 1–8, we could specifically amplify the AR mRNA in all three patients. Our RT-PCR data indicated that the reverse-transcribed AR cDNA and, consequently, the original fibroblast AR mRNA in the patients were intact and not affected by potential aberrant splicing events. We then controlled RT-PCR results by Northern blotting. While P3 showed at most a slightly reduced hybridization signal compared with the normal male controls, in P1 and P2 only very faint signals were present. This observation was well in accordance with the strikingly reduced AR mRNA transcript levels in P1 and P2 as quantified independently by LightCycler analysis. Taken together, the AR mRNA data supported the AR protein data and suggested the presence of potential mutations in the AR gene.

Lowered or completely absent expression of the AR may have different causes, among which have been published a complete [7] or partial [8] deletion of the AR gene, in-

sertions or deletions causing a frameshift leading to premature termination of translation further downstream [24], the direct introduction of a stop codon mutation [23], splice site mutations [10–12], or a reduced stability of the AR mRNA due to point mutations [5]. In all three patients, all eight exons of the AR gene could be amplified successfully by PCR using genomic DNA, resulting in DNA fragments of the expected length, thus excluding a potential deletion of the AR gene. While SSCA of the whole coding region of the AR gene revealed no indication for the presence of an AR sequence abnormality, direct sequencing exposed relevant mutations in P2 and P3. Both mutations are localized within or near the variable trinucleotide regions. While SSCA has been documented to be a highly sensitive tool for the screening of DNA sequence alterations in PCR-amplified conserved DNA fragments [19, 25, 26], its sensitivity is reduced for mutation detection in the variable trinucleotide regions. We therefore conclude that these parts of the AR gene must be sequenced if no mutation is detected within the conserved regions and AIS is highly suspected based on the clinical evaluation.

The 2-bp deletion at codon position 472 leading to a premature stop of translation in codon 499 in the P2 newborn has also been published previously by Thiele et al. [22] in an adult. The authors observed a complete AIS phenotype and absent ligand binding, reduced AR mRNA in RT-PCR, and a missing AR protein in cultivated GSF. This constellation mirrors exactly the findings of our patient, demonstrating a very high consistency of the molecular and clinical consequences due to this mutation.

The CAG (Gln) to TAG (stop) mutation detected in P3 is similar to, but not identical with, a mutation detected earlier by Zoppi et al. [23] in two siblings. In contrast to the affected codon position 59 in P3 corresponding to the second CAG in the CAG repeat, the siblings published by Zoppi et al. harbored their mutation in codon 60 corresponding to the third CAG. This difference is interesting, as the associated molecular consequences on the AR seem to differ. While we were not able to detect any specific androgen binding in P3, both fibroblast cultures published by Zoppi et al. showed very low but specific androgen binding. The authors had experimental evidence from transfection experiments in COS cells that this binding may have arisen from downstream initiation of translation of an AR at methionine 188. It may be suggested that the difference in AR translation could be affected by the exact location of the stop codon in the early CAG repeat. Interestingly, the pubic hair of P3 was clearly visible (Fig. 1b), suggesting at least partial androgen action. Therefore, it cannot be excluded that in our patient a minor amount of AR protein may exist in the genital target tissues below the detection level of our androgen-binding assay. However, neither Zoppi et al. [23] nor we were able to detect the resulting AR protein in the patients' fibroblasts using AR-specific antibodies.

The underlying mechanism of defective AR transcription and translation resulting in partial AIS in P1 despite the absence of a molecular abnormality in the entire AR

gene is most intriguing. Hypothetically, defective AR transcription in this patient leading to reduced AR protein may have been caused by a defective AR promoter due to a mutation or by a reduced cellular availability or dysfunction of an AR-promoter-interacting factor critical for the initiation of AR transcription. Interestingly, the AR promoter is known to be an important target for DNA methylation in prostate cancer leading to transcriptional silencing of the AR [27]. It may be speculated that an altered methylation pattern of the AR promoter may have contributed to reduced transcription in P1, but we have not investigated this hypothesis experimentally. Recently, a chromosomal abnormality with a t(X;5)(q13;p15) translocation affecting normal AR expression has been discussed as the underlying cause for partial AIS in two brothers [28].

In conclusion, our clinical and molecular genetic data on P1 noticeably support the concept that the diagnosis of AIS has to be established in some patients despite the absence of a mutation within the AR gene. While Adachi et al. [15] have previously reported the disease-causing role of AR coactivators in AIS, our new data clearly point to the existence of additional molecular mechanisms that primarily reduce the AR expression level in the genital target tissues. Because of the absence of a disruptive mutation, we believe that affected individuals are more likely to have partial instead of complete AIS.

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