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Complete androgen insensitivity caused by a splice donor site mutation in intron 2 of the human androgen receptor gene resulting in an exon 2-lacking transcript with premature stop-codon and reduced expression

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Abstract

Various mutations within the human androgen receptor gene have been documented to cause defective sexual differentiation in karyotypic male individuals. In this study, we report a previously undescribed point mutation at the donor splice-site of the second intron of the androgen receptor gene in a patient with a completely female phenotype. The sequence alteration was detected by single-strand-conformation-analysis-PCR and genomic sequencing. Applying competitive reverse transcribed PCR, cDNA sequencing and Western blotting, we could demonstrate considerable aberrations of structure and concentration of the transcript and its translation product in the patient's fibroblasts from the genital region. (1) In the transcript, exon 1 and 3 are directly linked to each other, the complete second exon is skipped. The mRNA predictively suffers a codon frame-shift in exon 3 associated with a premature termination between codons 598 and 599, leading to a truncated androgen receptor protein lacking any in vivo function. (2) Steady-state concentration levels of transcript and protein are abnormally low. Our observations highlight the influence of exon-flanking intron sequences on proper expression and function of gene products. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: AIS; Androgen receptor; Splice-site mutation; Transcription

1. Introduction

Androgen action represents the basis of normal sexual differentiation in males. In its target cells, androgen signalling is mediated by the androgen receptor (AR): This phosphoprotein forms a complex with the androgenic steroid (i.e. testosterone or dihydrotestosterone), homo-dimerizes and finally acts as a transcription factor on genes which provoke male-specific development [1–3]. The AR itself is encoded by a X-chromosomal gene subdivided into 8 exons [4]. It encodes three different domains: Parts of exon 1 for the transactivation of target genes, exon 2 and 3 for the regions involved in DNA-binding, and exon 4 to 8 for ligand binding [5].

Male pseudohermaphroditism is often caused by androgen insensitivity syndromes (AIS), a disorder which is based on the inability of the androgen dependent target tissues to respond to androgens. Mutations within the AR-gene have been proven to represent a common cause of AIS [6–8,12]. Interestingly, the clinical spectrum of this disorder is highly variable, reaching from infertility (in phenotypically unambiguous male individuals) over slight undervirilisation to completely female phenotype [9]. Additionally, in various cases, one defined point mutation causing a single

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amino-acid exchange often correlates with varying phenotypes [10,11]. Until today, more than 300 patients with mutations spread all over the coding region of the AR have been collected in an international data base [12]. Here, we present a patient with complete AIS bearing a so far undescribed point mutation.

2. Materials and methods

2.1. Patient

The patient with karyotype 46XY and complete AIS (type 5 according to the classification of Sinnecker et al. [9]) was studied. The patient has normal female external genitalia without signs of virilisation. No Müllerian structures could be demonstrated by ultrasound. The hormonal baseline values in serum were 34.8 pmol/l for estradiol and <0.35 nmol/l for testosterone. After hCG-stimulation (5000 IU HCG/m^2 body surface area, intramuscular) the patient displayed a testosterone concentration of 2.34 nmol/l after 72 h. Although the mother was found to be a heterozygous carrier of the mutation, the remaining family history was negative. Gonadectomy was performed at the age of 6 years, at this time gonadal tissue was preserved for cell culture.

2.2. Single strand conformation analysis-(SSC)-A

Genomic DNA from the patient and the mother was isolated from peripheral blood leukocytes by standard procedures[13]. The whole coding region of the AR gene including exon/intron boundaries was amplified by PCR in 14 individual segments using primers derived from published sequences [14]. Following successful amplification, each fragment was screened for variations employing PCR-SSCA as previously described [8,15]. Briefly, PCR-products were heat-denatured and electrophoresed on nondenaturating polyacrylamide(PAA)-gels. PCR-products which showed aberrant migration compared to normal controls were further analysed by direct sequencing using the Amersham-Pharmacia ALF Express automated sequencer (Pharmacia, Freiburg).

2.3. Cell culture, RNA-analysis, competitive RT-PCR and Northern blots

Biopsy specimens from both gonads were dissected mechanically and incubated in medium (DMEM-F12, 5% CO_2, 10% fetal calf serum, antibiotics) at 37°C to grow fibroblasts. Genital skin fibroblasts (GSF) obtained from the preputium of a normal prepubertal boy of similar age and gonadal fibroblasts (GoF) from a patient with a normal AR but a mutation in the 17ß-hydroxysteroid-dehydrogenase III (17ß-HSD)-gene served as controls. Cells of the second passage were used for AR-expression studies.

Whole-RNA from cultured GoF from the patient with complete AIS, normal control GSF and 17ß-HSD-control GoF was isolated using RNeasy™-columns according to the manufacturers protocol (Qiagen, Hilden). RNA was quantified photometrically (absorbency at 260 nm measured in a DNA/RNA-calculator from Pharmacia). To determine quality and integrity of RNA and to test quantification results, aliquots of 5 μl were electrophoresed on formaldehyde-denaturing 1% agarose gels (RNA-gels; [16]).

To characterise the influence of a mutation within the AR-gene on its transcript, we performed RT-PCRs: 1 μg of whole-RNA from the AIS-patient GoF, normal control GSF and 17ß-HSD-control GoF, respectively, was reverse transcribed by specific antisense priming following the manufacturers protocols (GibcoBRL, Eggenstein). Then, a specific intron-spanning fragment of the AR-transcript (target) stretching from nucleotide position 1653 (according to Lubhahn et al. [14]) in exon 1 to position 2845 in exon 4 was amplified by PCR. PCR-solutions consisted of 1.0 mM $MgCl_2$, 20 pmol sense-primer AR Is (5′-TGG ATG GAT AGC TAC TCC GG-3′) and antisense-primer AR IIa (5′-ACT ACA CCT GGC TCA ATG GC-3′) [18], 200 μM dNTPs, 1×PCR-buffer (pH 9.0) and 1U Ampli-Taq DNA-polymerase (Perkin-Elmer, Weiterstadt). Cycling conditions were: 75 s denaturation at 94°C, 90 s primer annealing at 60°C and 120 s primer extension at 72°C for 35 cycles. For sequencing of the RT-PCR product, we used a sense-primer specific for a sequence located about 92 bp upstream (from nucleotide position 1562 to 1582) of the ordinary sense primers' recognition-sequence in exon 1 of the gene (AR E1SVs: 5′-ACT TCA CCG CAC CTG ATG TG-3′) for amplification [8,15]. To screen the patients AR-mRNA for splicing-variants, we amplified a fragment spanning from the end of exon 1 (primer AR Is) to the initial part of exon 8 (primer AR E8a from position 4266 to 4287: 5′-GAG GAG TAG TGC AGA GTT ATA A-3′) including all exon boundaries. For the semiquantification of AR-transcription, we applied competitive RT-PCR: whole-RNA from patient GoF and both controls (normal GSF and 17β-HSD-control GoF) was first standardised for ubiquitous ribosomal protein L7 transcription as described (semiquantitative RT-PCR with modified primers; [18]). Then, each RNA-sample was mixed with an appropriately concentrated artificial RNA-standard (shortened target) and submitted to a RT-PCR as described above. Nondenatured PCR-products (target: 469 bp; standard: 346 bp) were electrophoresed on nondenaturating PAA-gels, silver-stained as previously described

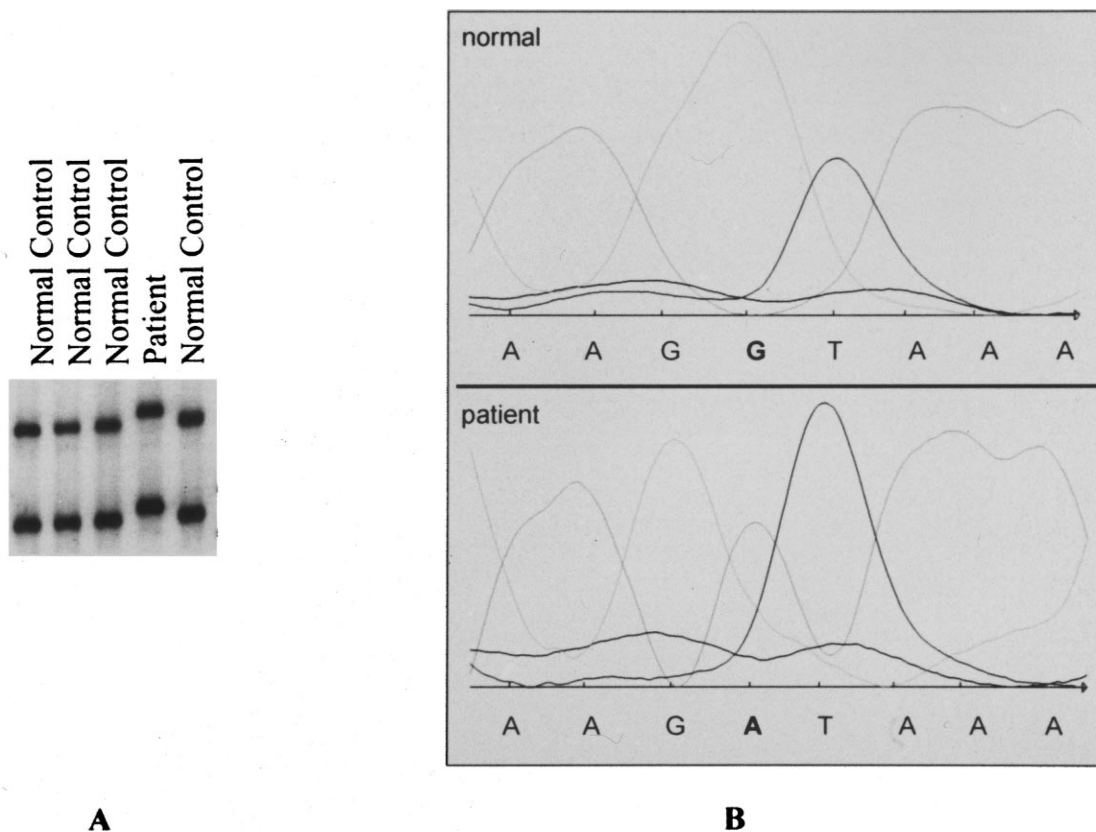

Fig. 1. SSC-A (A) and electropherograms of the genomic sequence (B) of exon 2 from PCR products from the patient and the normal control. A guanine to adenine substitution (Fig. 2B; bold letters) leads to the loss of the splice-donor site of exon 2. For further details see text.

[8,15] and finally quantified by computerised densitometry (ImageMaster^{TM}, Pharmacia). The artificial standard (competitor) was constructed by AR RT-PCR with composite-primers derived from published sequences as described [17,19–21]; additionally, the competitor was tailed with a T7-RNA-polymerase recognition-sequence at its 5′-end by the sense-composite-primer. Then, the product was in vitro transcribed, quantified and finally diluted to obtain working-stock aliquots for further usage in competitive RT-PCRs [20].

For Northern-blotting, 4 μg RNA per lane were loaded on RNA-gels and electrophoresed. Gels were equilibrated and blotted with 20×SSC onto positively-charged nylon membranes using a vacuum-blotter from Hoefer (Pharmacia). Digoxigenin-labeled AR-RNA-probes were generated by reverse-transcribed polymerase chain reaction (RT-PCR) using the protocols described above, in which the antisense-primer was tailed by a T7-RNA-polymerase recognition-sequence at its 5′-end. The product (469 bp) was electrophoresed on a 2% agarose-gel, isolated from the gel, purified by QiaQuick-columns (Qiagen, Hilden) and finally in vitro transcribed by T7-RNA-polymerase (Promega, Heidelberg) using digoxygenin-labeled rNTPs according to the respective manufacturers instructions. Northern-blot filters were initially prehybridized for 1–2 h and then hybridised over night with Dig-easy-Hyb (Boehringer-Mannheim, Mannheim) containing AR-RNA-probe at 68°C. Blots were washed twice for 5–10 min with 2×SSC/0.1%SDS at room temperature; stringent washings were performed at 68°C with 0.1×SSC/0.1%SDS for 2 × 15 min. Chemiluminescence signals of hybridised probes were developed following the "Digoxygenin users guide for Northern-blots" (Boehringer-Mannheim). Gels were stripped and reprobed with ß-actin DNA-probe as described above; however, hybridisation and washing-procedures were performed at 42°C.

2.4. Analysis of AR translation-products

Western-blots for AR-protein in the AIS-patient's GoF and normal control GSF were executed as described without further modifications [22,23]. In brief, patient gonadal fibroblasts and normal GSF were first lysed to isolate AR proteins from the crude cell-lysates by immunoprecipitation using the monoclonal antibody F39.4. Then, samples were separated by SDS-PAGE and electroblotted on cellulose-nitrate membranes. After incubation of the membranes with the primary antibody SP061 (directed against the

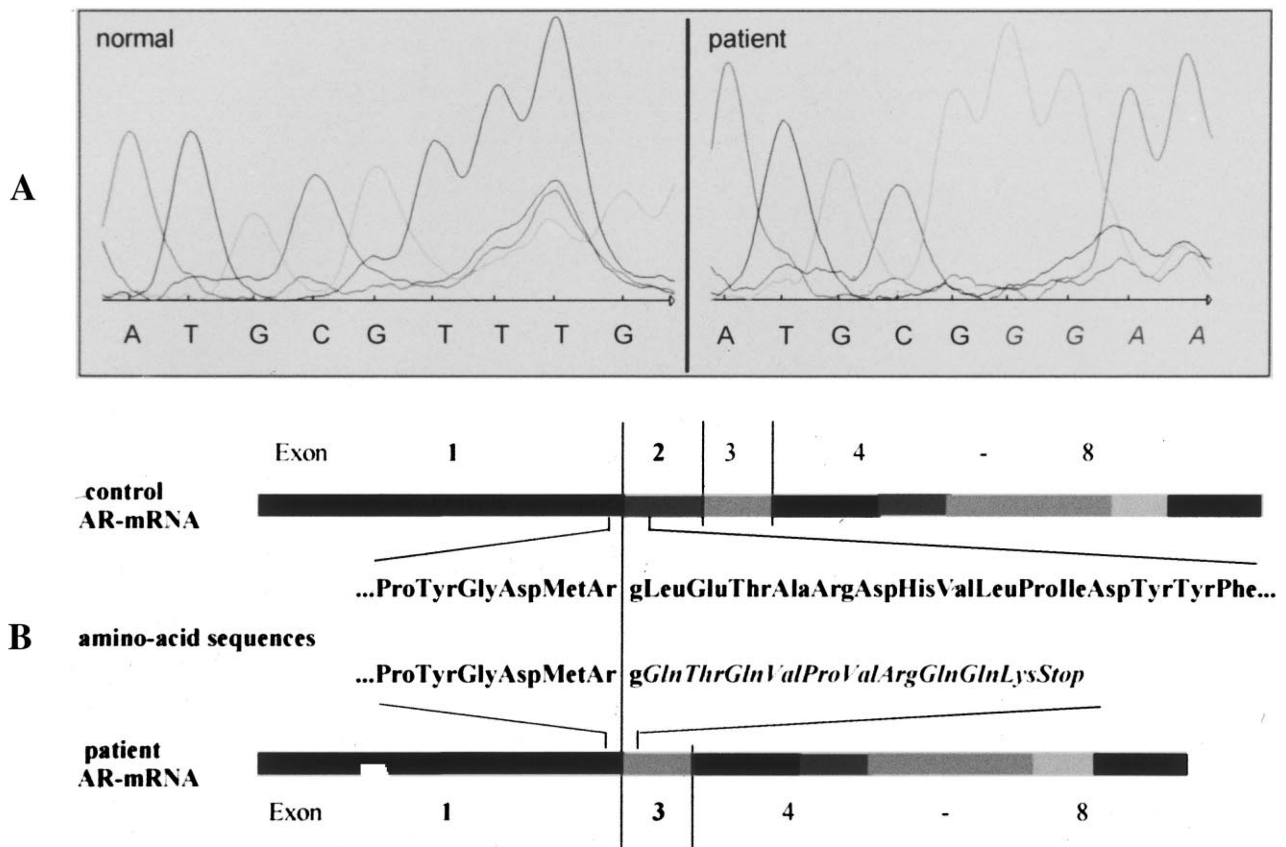

Fig. 2. Sequencing of the RT-PCR Product from the AR-mRNA in GoF from the AIS-patient and GSF from a normal control. The exon 2 sequence is not transcribed, instead the exon 3 nucleotide sequence (see grey coloured, italic typed sequence in the patient's electropherogram) is spliced to the exon 1 sequence. (A) Electropherograms of the AR-sequence neighbouring the connection between exon 1 and 2 (normal control) and exon 1 and 3 (patient). (B) Schematic illustration of mRNAs and amino-acid sequences at the linkage of exon 1-2 or 1-3 of control or patient respectively. Grey coloured italic letters represent a missense amino-acid sequence followed by a stop-codon in the patient, resulting from a codon frame-shift induced by the link of exon 1 to exon 3.

amino acids 301–320 of the AR [22]), a second incubation with an antirabbit-peroxidase conjugate was performed. AR-proteins were detected by chemiluminescence (specific anti-AR antibodies were a generous gift of Dr. A.O. Brinkmann, Erasmus University, Rotterdam). Androgen-binding assays were performed according to previous descriptions [23,24]. In brief, confluent cultures of GoF and GSF were incubated with media containing various concentrations of (^{3}H)R1881 in the presence (or absence) of a 200-fold molar excess of unlabeled ligand. After one hour at 37°C, an aliquot of 50 µl culture medium was taken for total-count determination. Cells were washed, lysed and submitted to liquid scintillation counting, while 100 µl served for protein determination. Results were finally evaluated by computerised Scatchard analysis.

3. Results

3.1. Mutation-screening of the AR-gene

PCR-amplification products of the AR gene from the patient and a normal control were screened for mutations by PCR-SSCA. Single strands of the second exon of the patient-AR gene showed a significantly altered migration within the PAA-gel in respect to the normal control (Fig. 1a). Sequencing of exon 2 resulted in the detection of a point mutation in the donor splice-site at the end of the exon; as shown in Fig. 1b, guanine is replaced by adenine at position 1998 (according to Lubhahn et al. [14]).

Applying reverse transcribed PCR (RT-PCR) on the patients AR-mRNA with primers AR Is and AR IIa revealed a product of 327 bp, substantially shorter than the wildtype product (479 bp) in both controls

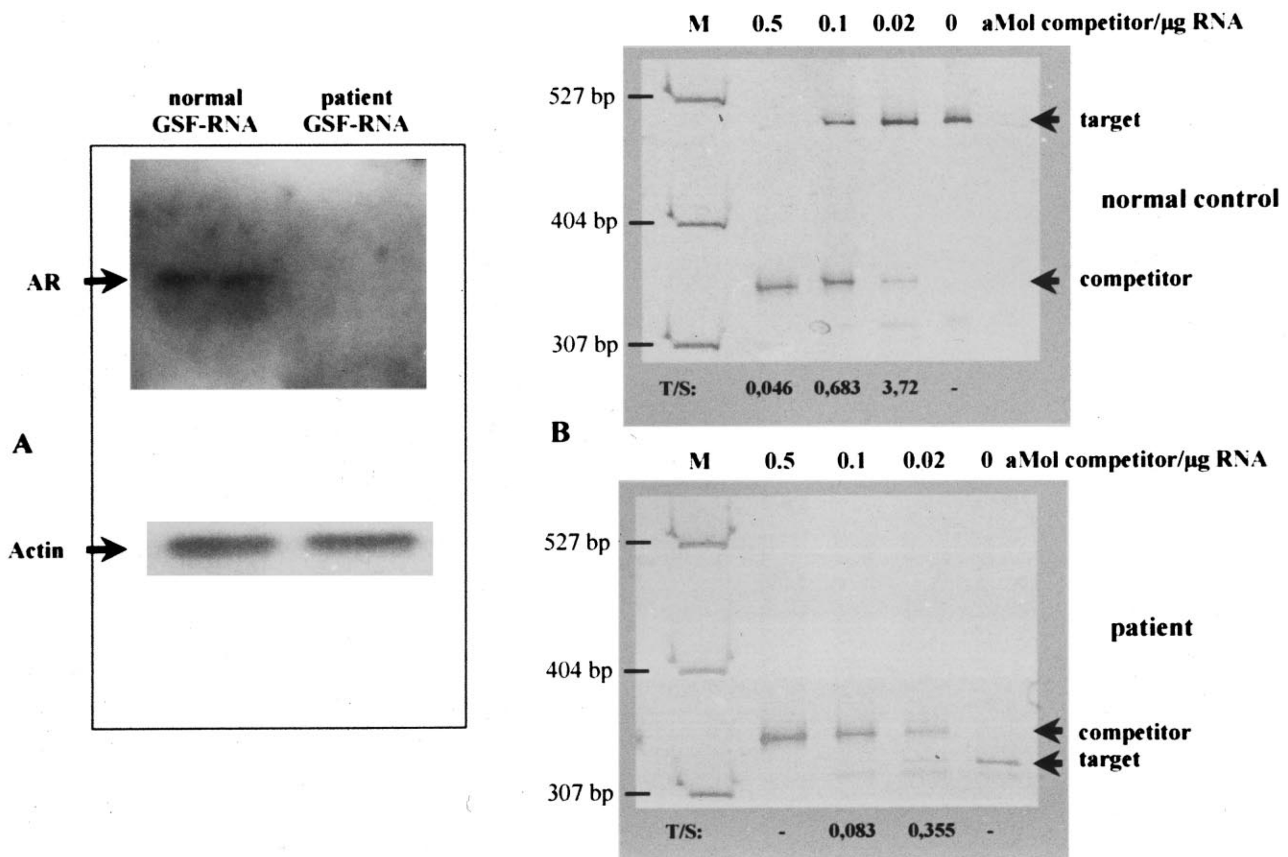

Fig. 3. Semiquantitative analysis of the AR-transcript concentration in GoF from the patient and controls. (A) Northern blot: 4 µg whole-RNA from the patient's GoF and the normal control's GSF (lane 1 and 2 respectively) was electrophoresed on an agarose-gel, blotted, hybridized to digoxygenin-labeled AR- and actin probes and developed by chemoluminescence-autoradiography. Similar β–actin signalling indicates equal RNA quantity and -quality in the patient's and control's lane. The normal control lane displays a AR-mRNA specific signal at 10.5 kb; in contrast, no specific AR-mRNA signal could be detected in the patient's sample. (B) Competitive RT-PCR: 1 µg whole-RNA from the patient's GoF and the 17β-HSD-control's GoF were each mixed with various concentrations of an artificially shortened AR RNA-standard (competitor; 346 ribonucleotides) and submitted to RT-PCR (wildtype product [target]; 479 bp). Products were electrophoresed on PAA-gels, silverstained and evaluated by densitometry. Target (T)- and standard (S)-product densities were compared as described in the text (T/S-values are indicated under the gels).The concentration of competitor in each PCR-sample is given at the top of the gels in attomole/µg RNA. M = molecular weight marker pBR 322-Msp 1 digest; fragment-lengths are given at the left side of the gels. As shown, the amplification product from the patient's RNA is shorter (327 bp) then the wildtype product (479 bp). The target concentration in the patient's samples is estimated to be between 11- and 15-fold lower than in the 17β-HSD control.

(normal control GSF and 17β-HSD-control GoF). The difference between patient- and wildtype product-length coincides with the length of wildtype exon 2 (152 bp). To amplify an AR-mRNA fragment which spans all exon-bounderies, RT-PCRs with primers AR Is and AR E8a where carried out. Again, the patient's PCR product was substantially shorter than the control sample. No other amplification products on patient- and both control's fibroblast RNA could be demonstrated. Direct sequencing of the RT-PCR product generated with primers AR E1SVs and AR IIa revealed the complete absence of the exon 2 sequence (Fig. 2a). Because of this alteration, the reading frame within exon 3 of the aberrant splicing-product predictively is shifted, resulting in a nonsense sequence of 10 amino acids followed by a premature stop-codon (Fig. 2b).

3.2. Northern-blot and competitive RT-PCR

To get information about the patient's AR-transcript concentration, we size-fractionated and blotted equal amounts of whole-RNA (4 µg) from the patient's GoF and normal control GSF and hybridised it with a RNA-probe specific to a sequence within the AR-transcript. As shown in Fig. 3a, no signal could be detected in the patients RNA, while the normal control demonstrated a slight but unambiguous AR-specific hybridisation band. Stripped and β-actin rehybridised blots, however, revealed similar β-actin signals

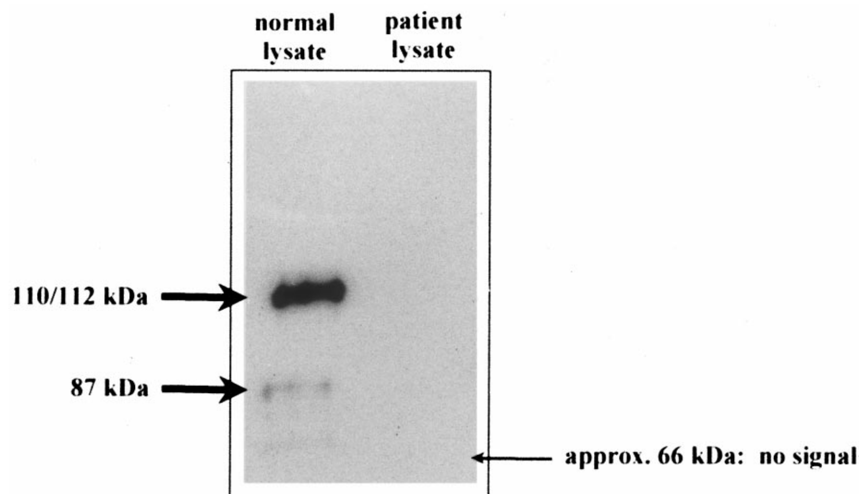

Fig. 4. Western blot with proteins from GoF from patient and normal control GSE Blots were first exposed to the anti-AR rabbit-antibody SP061. After a second incubation with an antirabbit-peroxidase conjugate, signals of AR-proteins were detected by chemiluminescence. Black arrows indicate normal AR-protein signals (110/112 kDa; 87 kDa), the grey arrow points at approx. 66 kDa, the region where a signal from the truncated patient-AR was expected. Here, no signal could be detected.

in both patient- and normal RNA-lane, indicating equal RNA-quantity and -quality.

As the absence of exon 2 in the patient's transcript may have hindered proper hybridisation of the probe, we performed competitive RT-PCRs to semiquantify even low amounts of the patient's AR-mRNA. After normalisation for ubiquitous ribosomal-protein L7 transcription by semiquantitative RT-PCR, 0.5, 0.1, 0.02 and 0 attomole RNA-competitor per 1 μg whole-RNA from patient GoF, normal control GSF, and 17β-HSD-control GoF were added. Samples were simultaneously reverse transcribed and finally submitted to PCR. After RT-PCR and PAA-electrophoresis, silverstained standard- (competitor) and target (AR-fragment) product-signals were evaluated by computerised densitometry: densities of target-(T) and standard-(S) product-bands were measured and compared to each other (resulting in a dimension-less number: T/S). In Fig. 3b, PAA-gels of competitive RT-PCRs on 17β-HSD-control GoF and patient GoF are illustrated. As indicated below the gels, the patient's T/S value at 0.02 attomole competitor/μg RNA (0.335) is nearly 11-fold lower than the corresponding T/S from the 17β-HSD control (3.617); at 0.1 attomole competitor/μg RNA however, the patient's T/S only reaches a 15-fold smaller value (0.083) than the control's (1.315). T/S values of competitive RT-PCRs on normal control GSF were slightly to 50% lower than the corresponding 17β-HSD-control GoF's values (not shown).

These results demonstrate that the target-product concentration in the patient samples is between 11- and 15-fold lower than in control GoF and 5- to 9-fold lower than in control GSF, indicating a markedly decreased AR-transcript concentration in the cells from the AIS-patient.

3.3. Translation and binding studies

To determine whether the results obtained at the transcription level of the AR reflect conditions at the protein level, we performed Western-blotting. As normal GSF proved to contain lower AR-mRNA levels compaired to 17β-HSD-control GoF (indicating a slightly lowered AR-protein content), we decided to relate patient-GoF and normal control GSF to demonstrate whether the AR-protein content in patient GoF could reach at least the AR-levels in the normal GSF control. Cell lysates were immunoprecipitated for AR-proteins with the antibody F39.4, separated on a SDS-PAA-gel, electro-blotted and incubated with the anti-AR antibody SP061. As shown in Fig. 4, two signals were detected in lane 1 containing normal GSF lysate, indicating the expression of the complete AR (strong signal at 110/112 kDa) and an additional AR-fragment (weak signal at 87 kDa) probably caused by downstream initiation of translation at Met^{189} [4]. However, the patient's sample in lane 2 did not display any signal, indicating that aberrant AR-protein expression is undetectable.

In order to obtain information about remaining androgen-binding capacities of the truncated patient-AR, we performed androgen-binding assays. While normal control cells displayed normal binding behaviour to (^{3}H)R1881 (B_{max} = 26,4 fmol/mg protein;

$K_d = 0.079$ nM), no specific androgen binding could be observed in the patient's gonadal fibroblasts.

4. Discussion

AR mutations are documented to be a common cause of AIS. Besides deletions, mostly point-mutations leading to amino acid substitutions are described (reviewed in Ref. [12]). These alterations cause more or less severe limitation or even total loss of AR-function depending on the difference of biochemical properties of mutated and wild-type AR. Some intronic mutations within or nearby splice-sites cause interesting aberrations of splicing process itself [24–28]. First, exons adjacent to the mutated region can be completely absent in mature mRNAs [27,28]. Second, mutations can lead to a mixture of mRNAs with either unaltered normal sequence, inserted nucleotides or absent exons [29]; this seems to depend on the activation of cryptic splice-sites. Here, we report a so far undescribed point mutation in the AR-gene, which obviously also leads to considerable structural changes of AR-transcript and -protein, causing complete AIS in the patient.

In the genomic DNA we detected an adenine–guanine transition at nucleotide position 1998 (according to Lubhahn et al. [14]) of the patient's AR-gene (Fig. 1b), which is the first nucleotide of the second intron. Thus, the wild type splice-donor site "GT(A)" at the 5′-end of this intron is altered to "AT(A)" and becomes inactivated. The resulting splicing process leads to a construct, in which the first exon is directly linked to exon 3; this is confirmed by sequencing the mutant's RT-PCR product (Fig. 2a). As no other splicing-products could be demonstrated by RT-PCRs enclosing all exon bounderies, we conclude that no alternative splicing, which theoretically could be initiated by cryptic splice-sites, occurred.

So far, different mechanisms of splice-site recognition and other presplicing events have been hypothesized [reviewed in Ref. [30]]. The "exon definition model" [31] give a possible explanation for the skipping of exons adjacent to mutated splice-site sequences. Following this model to describe normal AR-mRNA maturation, the splicosomal complex first adheres to the acceptor splice-site of intron 1. After finding the nearest downstream donor splice-site at the end of exon 2, it binds to it, defining the "overspanned" exon 2 as an individual information unit, which has to remain unchanged in the pre-mRNA for subsequent intron-outsplicing procedure. Finally, the splicosome complexes, "protects" exon 1 to 8 to merge these RNA-sequences and the splicing process is completed. As the patient's 5′-donor splice-site of intron 2 is destroyed and possible downstream cryptic donor splice-sites in this intron are not recognised, the splicosomal complex becomes unstable resulting in insufficient preservation of exon 2. Thus, the whole exon is skipped. Additionally, aberrant splicing of the AR pre-mRNA affects the coding sequence itself by causing a reading-frame shift at the connection between exon 1 and exon 3. The result is a sequence coding 10 missense amino acids residues followed by a premature stop-codon (Fig. 2b).

The splice-site mutation does not only affect AR-mRNA structure and information content, but also its concentration in the patients GoF, as we demonstrated by competitive RT-PCRs (Fig. 3b). We have to take in account that the amplification efficiency of shorter templates is normally higher than longer targets. Thus, compared with the longer normal template, the amplification of the aberrant template should be favored. However, the patient's aberrant target PCR-product appeared in considerably decreased concentrations compared to the product from the 17β-HSD-control GoF or the normal control GSF. We conclude, that in the patient's cells the AR-mRNA concentrations are at least 5- to 9-fold lower than in control GSF and even 11- to 15-fold diminished compared to control GoF. It has been reported by various authors (reviewed in Ref. [32]), that prematurely terminated proteins due to frame-shift or nonsense mutations are often preceded by reduced steady-state levels of the mutation-bearing mRNAs. The causes for this phenomenon are still not fully understood. On the one hand, accelerated degradation of mRNAs with nonsense-codons in nuclei or during nuclear translocation has been observed [33,34]. On the other hand, there is evidence for degradation of such mRNAs in cytoplasm: Lim et al. [35] demonstrated 1992 on germ cell lines of mice transfected with human β-globulin genes bearing premature stop-codons that the affected mRNA-precursors in the nuclei were properly processed, spliced and finally translocated. However, they became degraded in cytoplasm-seemingly in connection with the translational process—resulting in degradation products more stabile than full-length mRNA. These observations highlight the possibility that the observed low AR-transcript concentrations in the AIS-patient's gonadal fibroblasts are due to accelerated nuclear or cytoplasmatic degradation of the mutated mRNA.

Translation of wildtype AR-mRNA normally leads to two proteins of 110/112- and 87 kDa weight respectively—probably depending on the translation initiation at methionin^{1} or methionin^{189} [23]. In the Western blot shown in Fig. 4, the full length protein (110/112 kDa) from normal control GSF displays a strong signal, indicating pronounced translation start at Met^{1}. The patient's gonadal fibroblasts display no measurable amounts of AR-protein: neither a 110/112 or 87 kDa protein (wildtype), nor a shorter translation

product could be demonstrated. This result is well in accordance with the above described dramatically decreased transcript concentration within AIS-cells.

As demonstrated by androgen binding assays, the aberrant AR does not provide any androgen-binding capacity. Thus, we conclude that the described mutation does not seem to allow any alternative splicing with expression of the wild-type receptor. This consideration is supported by the failure to detect any alternatively spliced AR-mRNAs in the patients GoF and by the complete feminisation, indicating that no residual correctly spliced and functional AR-proteins exists.

Complete AIS in the patient presented can be sufficiently explained by the mutation itself, as it leads to a truncated, completely unfunctional AR-protein. However, concurrent to other findings, we demonstrate here that some mutations can lead to reduced transcription of the altered gene. Thus, in cases where the defect gene product still preserves a residual function, the disease may be the result of a summative effect of both, the functional defect, but also the reduced amount of translation product.

Acknowledgements

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