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Transcription of androgen receptor and 5α-reductase II in genital fibroblasts from patients with androgen insensitivity syndrome☆

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Abstract

Impaired virilisation during embryonic development and pubertal arrest in patients with androgen insensitivity syndromes (AIS) is usually caused by mutations in the androgen receptor (AR)- or the 5α-reductase II (5RII) gene. However, identical mutations may lead to strikingly different phenotypes. To investigate whether this may be caused by individually altered transcription rates in fibroblasts from the genital region (GF) from affected patients, we applied competitive reverse transcribed PCRs (competitive RT-PCR) targeting AR- and 5RII-transcripts. We could demonstrate that AR- and 5RII-mRNA concentrations in cells from patients with partial and complete AIS and missense mutations in the AR- or 5RII-gene are normal or only moderately lowered compared to equally aged normal controls. However, in a patient bearing a premature stop-codon in the AR-gene a considerably lowered AR-transcript level was detected. We conclude, that in patients with incomplete virilisation disorders due to missense mutations, transcription regulation of AR and 5RII generally follows normal patterns. Accordingly, the premature stop-codon found in one patient's AR-gene may rather cause reduced transcript stability than an impairment of transcription activity. Therefore, altered AR- and 5RII-transcription rates in fibroblasts from the GF do not seem to be the cause for the variable genotype-phenotype correlation in androgen insensitivity syndrome.

Keywords: Androgens; Androgen receptor; 5α-reductase II; Transcription regulation

1. Introduction

The development of the normal male genital tract is initiated by androgens via the androgen receptor (AR). The AR first binds the androgen testosterone (T) or dihydrotestosterone (DHT), then it forms homodimers and migrates into the nucleus. The AR-ligand complex binds to the promoter region of androgen sensitive target genes initiating together with other transcription factors the appropriate cell response [1–3]. During ontogenesis of the urogenital region, AR-bound T directly induces the virilisation of the Wolffian ducts, while in the mesenchymal cells of the sinus urogenitalis and genital folds, 5α-reductase II (5RII)-enzymes first reduce T to DHT. Hence, the AR-DHT complex induces the development of external genitalia [4]. Androgen insensitivity syndromes (AIS) are the most common cause of virilisation disorders in 46,XY individuals. They are based on the inability of androgen dependent target tissues to react on androgens. Most often AIS emerge due to function-impairing mutations in the AR-gene [5–9]; or the 5RII-gene [7,10,11]. However, in many cases one defined point mutation causing a single amino-acid exchange is linked with varying phenotypes [12–14]. For somatic mutations, this phenomenon can be explained by differences in mosaic mutation dispersion in androgen target tissues of affected patients [15,16]. However, the causes of individual phenotype variations in bearers of identical germline mutations in the AR still have not been elucidated satisfactorily. Additionally, in numerous cases *unmutated*, normal AR- and 5RII-genes can be found in individuals with AIS-similar phenotypes. One possible explanation for these observations is an impairment of transcription control of AR and 5RII. In patients with mutations,

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this could lead to a cumulative individual aggravation of virilisation disorders. Therefore, we aimed to study concentrations of AR- and 5RII-mRNAs within fibroblasts from genital skin or gonads (genital fibroblasts; GF) from a subset of 13 patients with mutations in the AR- or 5RII-gene displaying different degrees of undervirilisation.

2. Materials and methods

2.1. Cell culture

Biopsies from the genital tissue (gonads or genital skin) from 13 patients with androgen insensitivity of different severity (Table 1) and genital skin from 7 normal controls [17], see also Table 1) were dissected mechanically and incubated in media (DMEM-F12, 5% CO_2, 10% charcoal-stripped fetal calf serum, antibiotics) at 37°C to obtain fibroblasts. After the second passage, cells were stored in liquid nitrogen for later usage. Genital skin fibroblasts (GSF) from normal controls were denominated as 'NC' and cells from patients with virilisation disorders as 'ARD' (androgen resistance disorder). The labels were followed by an individual number and tailed by the passage-number.

After thawing, fibroblasts were first grown to 80% confluence and then pre-incubated in media without androgens for 48 h. Then cells were incubated in media containing 100 nM or 0 nM T for 48 h. Finally, fibroblasts were submitted to RNA-isolation.

2.2. RNA-analysis

Whole-RNA from fibroblasts was isolated using RNeasy™-columns following the manufacturers protocol (Quiagen, Hilden, Germany). RNA was quantified photometrically (absorbency at 260 nm measured in a DNA/RNA-calculator from Pharmacia, Freiburg, Germany). To determine quality and integrity of the isolated RNA and to test quantification results, aliquots of 5 μl were size-fractionated on formaldehyde-denaturing agarose gels (1%-RNA-gels) and analysed by ethidium-bromide-flourescence as described [18]. For exact standardisation of whole-RNAs from different cultures, we performed semi-quantitative analysis of ubiquitous ribosomal protein L7 transcription by kinetic RT-PCRs [19] as described [17,20]. After 21 PCR-cycles, samples were loaded onto 2% agarose gels, electrophoresed, stained with ethidiumbromide and analysed for equivalent amplification product amounts.

2.3. Competitive RT-PCR

Standardisation of cDNA-synthesis and PCR for all RNA-samples was made possible by the addition of

Table 1
Data on AIS-patients and normal controls[a]

AIS-type	Age	Mutated gene	Exon	Position	Mutation	Cells	Name
Partial, tend. masculine (2)	0 y. 11 m.	AR	3	aa 596	Ala → Asp	?	ARD 774
Partial, tend. masculine (2)	1 y. 5 m.	AR	4	aa 686	Cys → Arg	GSF	ARD 130
Partial, tend. masculine (2)	1 y. 6 m.	AR	4	aa 712	Leu → Phe	GSF	ARD 527
Partial, tend. masculine (2)	2 y. 1 m.	AR	4	aa 645	Ala → Asp	GSF	ARD 407
Partial, intermediate (3)	9 y. 7 m.	AR	3	aa 604	Asp → Tyr	GSF	ARD 82
Partial, tend. feminine (4)	9 y. 0 m.	AR	7	aa 840	Arg → His	GSF	ARD 2
Complete (5)	0 y. 4 m.	AR	7	aa 855	Arg → Cys	?	ARD 411
Complete (5)	2 y. 4 m.	AR	3	aa 608	Arg → Lys	?	ARD 534
Complete (5)	5 y. 8 m.	AR	1	aa 287	Glu → Stop	GSF	ARD 465
Complete (5)	18 y.	AR	6	aa 794	Phe → Ser	GoF; GSF	ARD 291
Complete (5)	35 y.	AR	5	aa 765	Ala → Thr	GoF	ARD 531
Partial, tend. masculine (2)	0 y. 7 m.	5RII	4	aa 196	Gly → Ser	GSF	ARD 249
Partial, tend. masculine (2)	5 y. 2 m.	5RII	4	aa 196	Gly → Ser	GSF	ARD 330
Partial, intermediate (3)	18 y.	5RII	1	aa 55	Leu → Glu	GSF	ARD 185
Normal male control	5 m.	–	–	–	–	GSF	NC 4
Normal male control	11 m.	–	–	–	–	GSF	NC 2
Normal male control	2 y.	–	–	–	–	GSF	NC 62
Normal male control	2 y. 7m.	–	–	–	–	GSF	NC 36
Normal male control	5 y. 10 m.	–	–	–	–	GSF	NC 24
Normal male control	12 y. 4 m.	–	–	–	–	GSF	NC 51
Normal male control	42 y.	–	–	–	–	GSF	NC 56

;>–>–>GSF>NC 56

[a] Patient and normal control data: AIS-type (according to ref. [29]) ordered for individual gravity, age at biopsy, information on the specific mutation, the origin of fibroblasts and nomenclature (see also ref. [17]). tend.: tendency to; y.: years; m.: months; aa: amino-acid (codon-number); GSF: genital skin fibroblasts; GoF: gonadal fibroblasts. In text and figures ARD- and NC-numbers are tailed by an additional number (e.g. – 2), which indicates the passage.

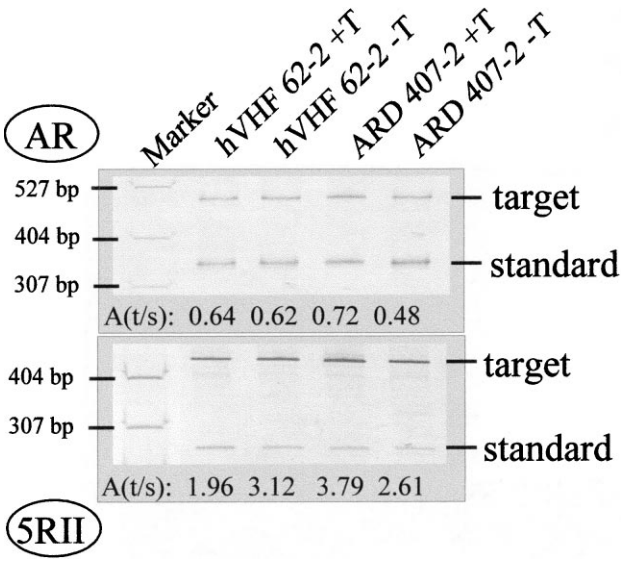

Fig. 1. Representative PAA-gels with silverstained products from competitive RT-PCRs on AR- and 5RII-transcripts in GSF from a partial-AIS patient ARD 407-2 and in GSF from the normal control NC 62-2 grown in media with 0- or 100 nM T. $+T$: cells grown with 100 nM T; $-T$: cells grown without T. Evaluation and results of all experiments see Fig. 2.

competitors (artificial standards with sequences complementary to the primers used for AR- or 5RII RT-PCRs). Competitors were constructed by RT-PCR with composite-primers derived from published sequences of AR- [21] and 5RII- [22] transcripts as described [17].

One microgram of whole-RNA samples were mixed with both (AR- and 5RII-) RNA-competitors of appropriate concentrations and antisense primers in each one Eppendorf-tube. Simultaneous reverse transcription of AR-/5RII-transcripts and competitors was achieved by specific anti-sense priming following the manufacturer protocols (*GibcoBRL*, *Eggenstein*, *Germany*). Then, 1 μl-aliquots from each cDNA-sample were submitted to separate PCRs using previously described primers [17]. Non-denatured PCR-products (AR-target: 479 bp, AR-standard: 324 bp; 5RII-target: 454 bp, 5RII-standard: 278 bp) were electrophoresed on polyacrylamide-gels (PAA-gels), silver-stained as previously described [6] and finally quantified by computerised densitometry (ImageMaster™, *Pharmacia*). Densities of target- and standard- (competitor) bands were compared to each other resulting in a dimension-less number: A(t/s). A(t/s) values served further as relative target-concentration equivalents. The accuracy of the method has been tested by us previously [17]. We have demonstrated that at least up to 4-fold differences in AR- and 5RII-mRNA concentrations can be described reproducibly using one competitor concentration. In assays on samples containing constant target-amounts, standard deviations did not exceed 10.2% for AR- and 14.8% for 5RII semi-quantitative competitive RT-PCRs compared to the mean values (set to 100%). This was found to be reliable for semiquantitations of AR- and 5RII-transcripts [17].

3. Results

3.1. AR- and 5RII-transcription in GF with mutations in the AR-gene

For the examination of AR- and 5RII-transcript concentrations in GF from AIS-patients with missense mutations (Table 1) in the AR-gene and in normal control genital skin fibroblasts (GSF; see also Table 1) 0.2 attomole AR- and 0.01 attomole 5RII-competitor were added per μg RNA. When L7-transcription in all samples was found to be similar, competitive RT-PCRs were performed. Representative PAA-gels with size-fractionated and silverstained AR- and 5RII-target and -standard amplificates and their corresponding $A(t/s)$-values generated on ARD 407-2 and a normal control are shown in Fig. 1. As shown in Fig. 2A–C, $A(t/s)$-values for fibroblasts from patients with partial AIS are similar or even 1.9 to 2.3-fold (ARD 527-2 [AR]) or 4-6-fold (ARD 2-2 [5RII]) higher than in equally aged normal controls. This proves an undiminished transcription activity. Only ARD 774-2 cells display a moderately lowered 5RII-transcription compared to the normal control (≈ 45–60%). In contrast, as shown in Fig. 2D–F, AR- and 5RII-mRNA concentrations in fibroblasts from patients with complete AIS are continuously lowered: mean AR-transcript concentrations in GSF from the patient ARD 291-3 achieve ≈ 35–44% of those in normal GSF, while AR-transcription in gonadal fibroblasts (GoF) from ARD 291-3 and ARD 531-3 appears to be slightly higher. GF from the patients ARD 411-2 and ARD 534-2 display AR-transcript concentrations of $\approx 50\%$ and 40–55%, respectively, compared to their control. 5RII-transcription in GF of 3 patients with complete AIS (ARD 291-3, ARD 531-3 and ARD 411-2) is absent or too low to be measurable by our method. In ARD 534-2 cells, a 10–14-fold lower 5RII-mRNA-concentration was measured than in the respective normal control. A characteristic dependence of AR- or 5RII-transcript concentrations in AIS-patient GF on mutation site or T-supplementation in media could not be determined.

AR- and 5RII-mRNA concentrations in GSF from a patient (ARD 465-2) with complete AIS and a nonsense mutation in the AR-gene were measured by competitive RT-PCRs using descending AR- and 5RII-competitor concentrations. As shown in Fig. 3, the AR-transcript level was 13-fold lower than in the normal control. 5RII-transcription was not reduced (not shown).

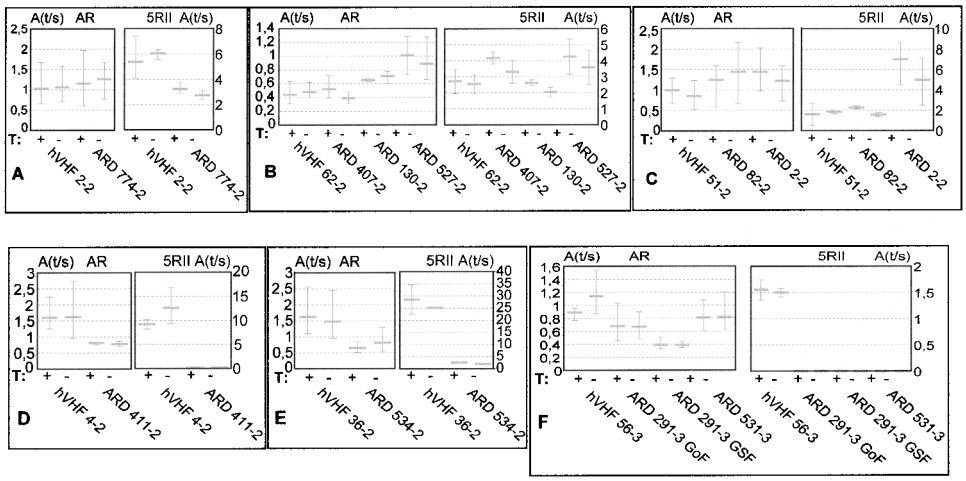

Fig. 2. AR- and 5RII-transcription in fibroblasts of the GF from AIS-patients with missense mutations in the AR-gene. Incubation in 100 nM T (+ *T*) or without T (− *T*). Measurements were made by competitive RT-PCRs (0,2 attomole AR- and 0,01 attomole 5RII-competitor per μg RNA). The experiments are divided into the series A–F (per series 1–3 patients and one normal control of similar age were analysed simultaneously). For each series, the left graph depicts AR-, the right one 5RII-transcription (in *A*[*t*/*s*]). In the graphs, mean values (horizontal bars) and the highest positive and negative deviations (error bars) are depicted of 3- (AR; all series) or 2–3 measurements per culture (5RII; 2 assays in series B, E, F and 3 assays in series A, C, D). In series A–C patients with partial AIS, in series D–F patients with complete AIS are shown (Table 1). GoF: gonadal fibroblasts; GSF: genital skin fibroblasts.

3.2. 5RII- and AR-transcription in GF with mutations in the 5RII-gene

GSF from 3 patients with mutations in the 5RII-gene (Table 1) were analyzed for their 5RII- and AR-transcription by competitive RT-PCRs. An examination similar to the analysis of AR-transcription in ARD 465-2 GSF (Fig. 3) was applied for 5RII-mRNA semiquantitation using 0,003-, 0,01- and 0,03 attomole 5RII-competitor/μg RNA. At all competitor concentrations, patients showed lowered 5RII-transcription compared to the normal control: at 0.01 attomole competitor/μg RNA, ARD 330-2 ($A[t/s] = 1.5$) achieved 20.5% and ARD 249-2 ($A[t/s] = 3.0$) reached 41% of the 5RII-transcript levels found in NC 2-2 cells ($A[t/s] = 7.3 = 100\%$). In cells from ARD 185-2, no 5RII-transcription could be determined. AR-mRNA concentrations in GSF from all 3 patients were normal (not shown).

4. Discussion

Mutations in the AR- and the 5RII-gene are known to cause a broad spectrum of phenotypic variations [7,11,23]. These variations may not be exclusively caused by the mutations themselves but may also be influenced by altered transcription rates. In the investigations presented here, however, missense mutations in the AR-gene associated with partial AIS of different severity were not associated with diminished transcription of AR or 5RII in fibroblasts of the GF (Figs. 1 and 2A–C). Nevertheless, the relatively high variations of 5RII-transcript concentrations compared to the normal controls (up to 4-6-fold higher $A(t/s)$-values as shown in Fig. 2) may reflect the wide individual variations of 5RII-transcription found by us previously in normal GSF [17]. We conclude that in patients with partial androgen insensitivity, the generation of their phenotype is not predominantly influenced by changes of AR- or 5RII-transcription rates. T did not exhibit a characteristic effect on AR- and 5RII-mRNA-levels in patient and normal GF. This fits with the observation of undiminished AR- and 5RII-transcription in partial-AIS patient cells: If a mechanism of androgen-driven AR- and/or 5RII-transcription regulation in GF existed, it should be disabled by mutations in the AR-

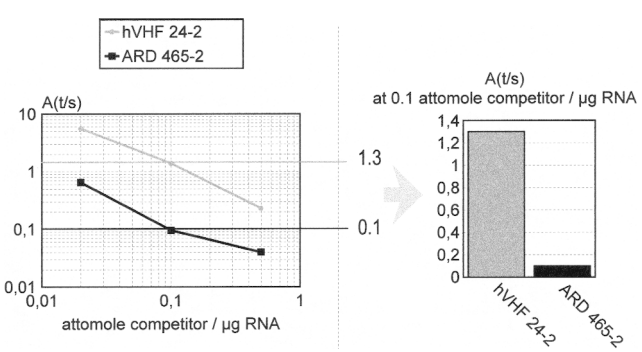

Fig. 3. AR-transcription in fibroblasts from patient ARD 465-2 with a nonsense-mutation in the AR-gene and a normal control (NC 24-2). Measurements by competitive RT-PCRs with declining competitor concentrations: 0,02-, 0,1- and 0,5 attomole/μg RNA. In the left graph, mean A(t/s)-values of 3 competitive RT-PCRs per probe are depicted logarithmically as a function of competitor concentration. Mean A(t/s)-values at 0,1 attomole competitor/μg RNA are shown in columns in the right graph.

gene. Consequently, one would expect altered AR- and/or 5RII-transcription. This did not occur. These findings are in accordance with recent studies, which demonstrate that androgens do not control transcription of AR [17,24] and 5RII [17] in normal GSF. In contrast, fibroblasts with missense mutated AR-genes from donors with complete AIS displayed slightly to moderately lowered AR-mRNA levels compared to their normal control. With one exception (ARD 534-2), 5RII-transcription could not be measured in those cells (Fig. 2D–F). 5RII-transcription in cells with missense mutation in the 5RII-gene also displayed decreased 5RII-transcript levels in comparison to the normal control. This indicates an influence of complete-AIS associated AR-mutations and 5RII-mutations on AR- and 5RII-transcription. Seemingly, this contrasts the results obtained on GF from partial AIS patients. However, it remains questionable to assume an influence of AR- and 5RII-transcription rates on the AIS phenotype additional to the mutation-caused protein function deficiency. First, the AR-mRNA level decrease in GF from complete-AIS individuals is relatively low. The transcription achieves between 35% and 55% (GSF) or up to 72–91% (GoF) of transcription in normal control GSF making a marked effect on genital ontogenesis improbable. Second, 5RII-transcription has not been found in gonadal tissue so far [25]. As the examinations of ARD 531-3 were applied on GoF and the exact origin of GF from ARD 411-2 and ARD 534-2 is unknown, the lack of 5RII-transcription in these cells can not be assigned doubtlessly to be abnormal. However, for ARD 291-3 we could not find 5RII-transcription in GoF and GSF. In our previous study on GSF from normal male donors [17], we not only detected wide individual variations, but also a dramatic age-dependent decline of 5RII-transcription (see also normal control Fig. 2f). The donor of ARD 291-3 cells was already 18 years old when tissue was preserved. Therefore, the lack to detect 5RII-transcription in ARD 291-3 GSF may reflect an age-specific and individually low – or only moderately lowered – transcription rate with minor importance for the formation of the complete AIS phenotype. This age effect could also explain apparently lowered 5RII-mRNA concentrations observed in GSF from patients with a mutation in the 5RII-gene: ARD 330-2 is 4 years and 7 months older than ARD 249-2; correspondingly, the $A(t/s)$-value indicating the 5RII-mRNA level in its GSF is 2-fold lower than that from ARD 249-2. However, both patients share mutation and phenotype (Table 1). These details do not rule out a possible effect of mutations on 5RII-transcription activity, but they point to the ambiguity of this hypothesis and – sequentially – of an eventual influence of variable 5RII-transcript levels on the AIS-phenotype.

In contrast to missense mutations, AR-transcription in a patient (ARD 465-2) with a nonsense-mutation in the AR-gene was dramatically diminished (Fig. 3). Lowered transcript-levels reported on genes with premature termination codons [26] are probably the result of an accelerated degradation rate of the affected mRNA [27,28]. Accordingly, we assume that impaired stability is the cause for the low AR-transcript levels in ARD 465-2 cells. However, the complete AIS phenotype is explained exclusively by the severe alteration of AR-translation due to the inserted stop-codon within the first exon itself. In contrast, we believe that those missense mutations in the AR- and 5RII-gene which are associated with diminished AR- (and eventually 5RII-) transcript levels (Fig. 2D–F) may be the result of more complex physiological processes, such as an androgen-driven transcription regulation apparatus restricted to phases during fetal development or by interactions dependent on natural tissue-compounds and mediators. These presuppositions will have to be investigated in the future.

We conclude that concentrations of missense mutated AR- (and possibly 5RII-) transcripts are only moderately diminished in cells from donors with a complete AIS phenotype. However, moderate differences of AR- and 5RII-mRNA levels can not be assigned to have a doubtless influence on the individual virilisation phenotype. Therefore, alterations of AR- and 5RII-transcription rates in GF do not appear to cause the variable genotype–phenotype correlation in AIS.

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