Tumor and Stem Cell Biology

Evidence of Limited Contributions for Intratumoral Steroidogenesis in Prostate Cancer

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Abstract

Androgen-deprivation therapy for prostate cancer (PC) eventually leads to castration-resistant PC (CRPC). Intratumoral androgen production might contribute to tumor progression despite suppressed serum androgen concentrations. In the present study, we investigated whether PC or CRPC tissue may be capable of intratumoral androgen synthesis. Steroidogenic enzyme mRNAs were quantified in hormonally manipulated human PC cell lines and xenografts as well as in human samples of normal prostate, locally confined and advanced PC, local nonmetastatic CRPC, and lymph node metastases. Overall, the majority of samples showed low or absent mRNA expression of steroidogenic enzymes required for *de novo* steroid synthesis. Simultaneous but low expression of the enzymes CYP17A1 and HSD3B1, essential for the synthesis of androgens from pregnenolone, could be detected in 19 of 88 patient samples. Of 19 CRPC tissues examined, only 5 samples expressed both enzymes. Enzymes that convert androstenedione to testosterone (AKR1C3) and testosterone to dihydrotestosterone (DHT; SRD5A1) were abundantly expressed. AKR1C3 expression was negatively regulated by androgens in the experimental models and was increased in CRPC samples. Expression of SRD5A1 was upregulated in locally advanced cancer, CRPC, and lymph node metastases. We concluded that intratumoral steroid biosynthesis contributes less than circulating adrenal androgens, implying that blocking androgen production and its intraprostatic conversion into DHT, such as via CYP17A1 inhibition, may represent favorable therapeutic options in patients with CRPC. *Cancer Res; 70(3); 1256–64. ©2010 AACR.*

Introduction

Despite current early detection methods and surgical and radiotherapeutic treatment options, many prostate cancer (PC) patients still present with unresectable stages of disease (1). Since 1941, the mainstay of treatment of advanced PC is focused on suppression of intraprostatic testosterone and dihydrotestosterone (DHT) actions. Nowadays, androgen deprivation therapy is based on lowering the luteinizing hormone (LH)–induced testicular testosterone production through LH-releasing hormone agonists (chemical castration) with or without antiandrogens that block the androgen receptor (AR). Growth inhibition is initially achieved in the majority of patients. However, eventually all patients develop hormone-refractory or castration-resistant PC (CRPC), marked by an increase in prostate-specific antigen (PSA) and progression of the tumor (2). Second-line treatment with docetaxel chemotherapy combined with prednisone is only temporarily effective with a small survival benefit (3). Due to this untreatable stage of disease, PC is still estimated to be the cause of 28,660 deaths in the United States in 2008 alone (4).

Several hypotheses underlie the occurrence of CRPC, such as the selective outgrowth of androgen-independent clonal cell populations caused by activation, suppression, or fusion of genes and AR signaling pathway–related causes, encompassing ligand-independent AR activation, AR hypersensitivity due to AR overexpression, and ligand promiscuity due to AR mutations (5, 6). More recently, intratumoral conversion of adrenal androgens and *de novo* steroid synthesis have been brought forward as potential causes of tumor progression (7–9). The presence of active AR in CRPC samples and the reported high intratumoral testosterone and DHT concentrations in CRPC patients with castrate serum androgen levels support the concept of intratumoral conversion of steroidal precursors (8, 10). Recent publications have put renewed emphasis on this intratumoral steroidogenesis by showing the previously unknown expression of steroidogenic enzymes in normal prostate and PC tissue (8, 9, 11, 12), as well as a differential expression pattern between the various tumor types and the normal prostate gland (8, 9). Furthermore, conversion of the radiolabeled steroid precursor acetic acid into DHT has been shown to occur *in vitro* in LNCaP cells and *ex vivo* in CRPC cells (7). The potential upregulation of steroidogenic enzymes in CRPC and the resulting local testosterone and DHT production may account for the observed intratumoral androgens in levels sufficient to activate the AR (7, 8,

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Figure 1. Simplified scheme of the classic steroid biosynthetic pathway. The depicted steroidogenic enzymes and cofactors are necessary for *de novo* synthesis of androgens from cholesterol and were investigated in this study. *CYP,* cytochrome P450; *HSD,* hydroxysteroid-dehydrogenase; *CYB5,* cytochrome b_5; *AKR,* aldo-keto-reductase; *SRD,* 5α-reductase.
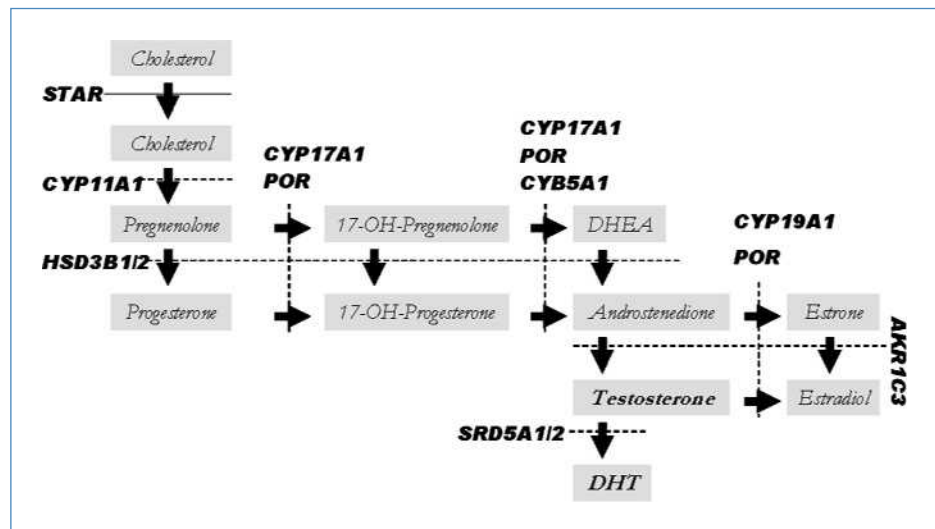
10, 13). Consequently, such a mechanism would require new therapeutic modalities that aim to block intratumoral steroidogenesis.

De novo steroid production has been thought to be confined to a few organs: the gonads, the adrenal cortex, and the placenta. Steroidogenesis is initiated by the transport of cholesterol through the mitochondrial membrane, for which the steroid acute regulatory protein (STAR) is obligatory (Fig. 1). Once cholesterol has entered the mitochondria, it can be converted into pregnenolone by desmolase, also known as cytochrome P450 side-chain cleavage enzyme (CYP11A1). The Δ5-steroid pregnenolone can subsequently be converted through 17-hydroxylase and 17,20-lyase activities [both encoded by CYP17A1, together with cofactors cytochrome P450 oxidoreductase (POR) and cytochrome b_5 (CYB5A1)] and through 3β-hydroxysteroid dehydrogenase activity (by either of the isoenzymes HSD3B1 or HSD3B2) to form androstenedione (14, 15). Further metabolism of androstenedione can take place in peripheral tissues, such as the male reproductive tract. Here, several 17β-hydroxysteroid dehydrogenase enzymes are able to convert androstenedione to testosterone, of which the type 5 enzyme (AKR1C3 or HSD17B5) is the most important isoform in the prostate gland (16). Androstenedione and testosterone can be aromatized into estrogens by CYP19A1. The two 5α-reductase isoforms, SRD5A1 and SRD5A2, are able to metabolize testosterone to DHT. The type 2 reductase is thought to be the most important isoform for intraprostatic conversion (16).

To study the capability of human PC to synthesize androgens *de novo* as well as to convert adrenal androgens locally, we measured steroidogenic enzyme expression in a large set of hormonally manipulated experimental models of PC and in patient material from normal prostate, local PC, lymph node metastases, and transurethral resection of the prostate (TURP) tissues. In all samples, the expression of key enzymes required for *de novo* synthesis of androgens was low or absent and was not affected by androgen ablation therapy. The enzymes required for testosterone and DHT production from androstenedione were abundantly expressed. Their expression was affected by androgen levels and could be related to PC progression.

Materials and Methods

In vitro *cultures and tumor-bearing mice.* The investigated cell lines and xenografts have been characterized previously (17–21), as summarized in Table 1. The PC cell

Table 1. Prostate cancer cell line and xenograft characteristics

	Androgen dependence	AR status	Origin
Cell lines			
LNCaP	Responsive	AR+	Lymph node
VCaP	Responsive	AR+	Bone
DuCaP	Responsive	AR+	Dura
PC346C	Responsive	AR+	PC346
Xenografts			
PC82	Dependent	AR+	Prostate
PC295	Dependent	AR+	Lymph node
PC310	Dependent	AR+	Prostate
PC346	Responsive	AR+	TURP
PC346B	Responsive	AR+	TURP
PC374	Responsive	AR+	Skin
PC133	Independent	AR−	Bone
PC135	Independent	AR−	Prostate
PC324	Independent	AR−	TURP
PC339	Independent	AR−	TURP
PC346I	Unresponsive	AR+	PC346
PC346BI	Unresponsive	AR+	PC346B
PC374F	Unresponsive	AR+	PC374

Table 2. Patient characteristics

	First series					Second series	
	Normal prostate	**Locally confined PC**	**Lymph node metastases**	**Locally advanced PC**	**CRPC**	**Locally advanced PC**	**CRPC**
No. patients, unique	17	11	16	21	10	4	9
Age at diagnosis [median (range)], y	62 (54–72)	62 (56–70)	66 (52–71)	63 (46–68)	62 (53–68)	73 (67–81)	68 (50–86)
Gleason score, *n*							
6		6	0	3	1	2	2
7		3	4	4	5	0	1
8		1	9	13	3	1	2
9–10		1	3	1	1	1	4
Epithelium in tissue [average (range)], %	72 (60–80)	83 (70–100)	90 (60–100)	87 (70–100)	88 (70–100)	85 (70–90)	71 (30–90)
Cancer in tissue [average (range)], %	0	89 (70–100)	100	98 (90–100)	97 (90–100)	88 (80–100)	94 (80–100)

lines LNCaP and PC346C were grown in DMEM/F-12 and VCaP and DuCaP in RPMI 1640, all in the presence of 5% FCS and penicillin/streptomycin (Invitrogen), until the start of the experiment, followed by two consecutive 36-h periods in androgen-deprived medium containing 5% dextran-coated charcoal-stripped FCS. Subsequently, cells were incubated with 1 nmol/L of the synthetic androgen R1881 (NEN) or ethanol vehicle. After 8 h, the medium was removed and cells were frozen and stored at −80°C.

Thirteen established xenografts of prostate carcinomas were grown in nude mice. The xenografts were designated as being androgen dependent, androgen responsive, androgen independent, or androgen unresponsive on the basis of their (in-)ability to proliferate in castrated nude mice (17). Xenografts were collected from intact or 7- to 14-day-castrated male mice, snap-frozen, and stored at −80°C.

Patient samples. Patient samples were collected from patients operated within the Erasmus MC between 1984 and 2001, after approval from the local medical ethics committee. Tissues included samples from radical prostatectomy specimens of locally confined prostate carcinoma, lymph node dissection of metastases, and transurethral resection of locally advanced prostate cancer (TURP) or of CRPC (Table 2). CRPC samples were obtained from patients with urinary obstruction due to locally progressive disease during androgen deprivation therapy. A second series of four locally advanced PC (TURP) and nine CRPC samples was collected to verify primary data (Table 2). Sections from tumor areas and normal tissues were snap-frozen in liquid nitrogen and stored at −80°C. H&E-stained slides of the frozen sections were scored independently by two pathologists for percentage of tumor tissue and Gleason score. In each slide, the percentages of normal epithelial, stromal, and tumor nuclei were scored. Abundant presence of inflammatory cells was recorded. Normal prostate was defined as benign prostate tissue containing more than 60% glands. Tumor tissue was used for subsequent analyses if >70% of cells were tumor.

RNA isolation and quantitative reverse transcriptase-PCR. RNA from prostate specimens, xenografts, and cell lines was isolated using RNAbee reagent as described by the manufacturer (Tel-Test, Inc.). The reverse transcriptase reaction was done with 1 μg of RNA and oligo T12 primer and preincubated for 10 min at 70°C. First-strand buffer, DTT, deoxynucleotide triphosphates, RNAsin, and Moloney murine leukemia virus reverse transcriptase (Promega Benelux B.V.) were added and incubated for 1 h at 37°C. After this, the reaction was kept for 10 min at 90°C and samples were immediately frozen thereafter.

Gene expression in the cell lines and xenografts was analyzed in an ABI Prism 7900 Sequence Detection System. Primer and probe sequences are depicted in Supplementary Table S1. Each assay was tested beforehand for human cDNA specificity and did not detect human DNA or murine cDNA equivalents. PCR efficiency was checked by cDNA dilution curves, and efficiency exceeded 90% for all assays. The real-time PCR reaction was done in a volume of 12.5 μL, containing 20 ng cDNA, 2× TaqMan Universal Master Mix (Applied Biosystems), 300 nmol/L primers, 100 nmol/L probe (Biolegio), and H_2O. In case of validated TaqMan Gene Expression Assays (Applied Biosystems), a 1:50 volume of the primer-probe mix was used. Positive controls consisted of cDNA of human placenta (HSD3B1 and CYP19A1), normal prostate gland (SRD5A2), normal adrenal cortex (HSD3B2), or the steroid-secreting adrenocortical cell line H295R (other assays). Expression was calculated relative to the average of threshold cycles (Ct) of two housekeeping genes, *HPRT1* and *GAPDH*, using the δCt method. Ct values >40 were considered as no expression.

The patient samples were analyzed likewise in an ABI Prism 7500 FAST Sequence Detection System using a total of 15 μL reaction volume. Other human tissue and cell line controls were added to compare the prostate samples to tissues with presumably low mRNA expression of steroidogenic enzymes. Because of the low expression of HSD3B1 and

CYP17A1 mRNA, additional assays were done using commercially obtained primer-probe combinations (Applied Biosystems). The PCR efficiency of these assays was 100% ($r^2 >$ 0.995) in the Ct range 26.0 to 36.7 and 22.3 to 39.6 for HSD3B1 (own design and Applied Biosystems, respectively) and 22.7 to 38.5 and 23.4 to 37.4 for CYP17A1. Expression was calculated relative to the expression of the housekeeping genes *GAPDH* and *HMBS*. For the quantitation of AR and HMBS mRNA, the reaction mix included SYBR Green PCR Master Mix (Applied Biosystems) and a dissociation stage was added to the PCR program to check for assay specificity.

Statistics. The effect of hormonal manipulation on steroidogenic enzyme expression in the cell lines and xenografts was analyzed using paired Student's *t* tests on relative values. Expression in patient samples was analyzed using Kruskal-Wallis tests and post hoc Dunn's multiple comparison tests for multiple groups or Mann-Whitney *U* tests for two groups. Correlation between gene expressions was calculated using Spearman's correlation coefficient with a Bonferroni-Holm correction for multiple testing. Analyses were carried out using GraphPad Prism version 5.01 (GraphPad Software). $P < 0.05$ was considered to be statistically significant.
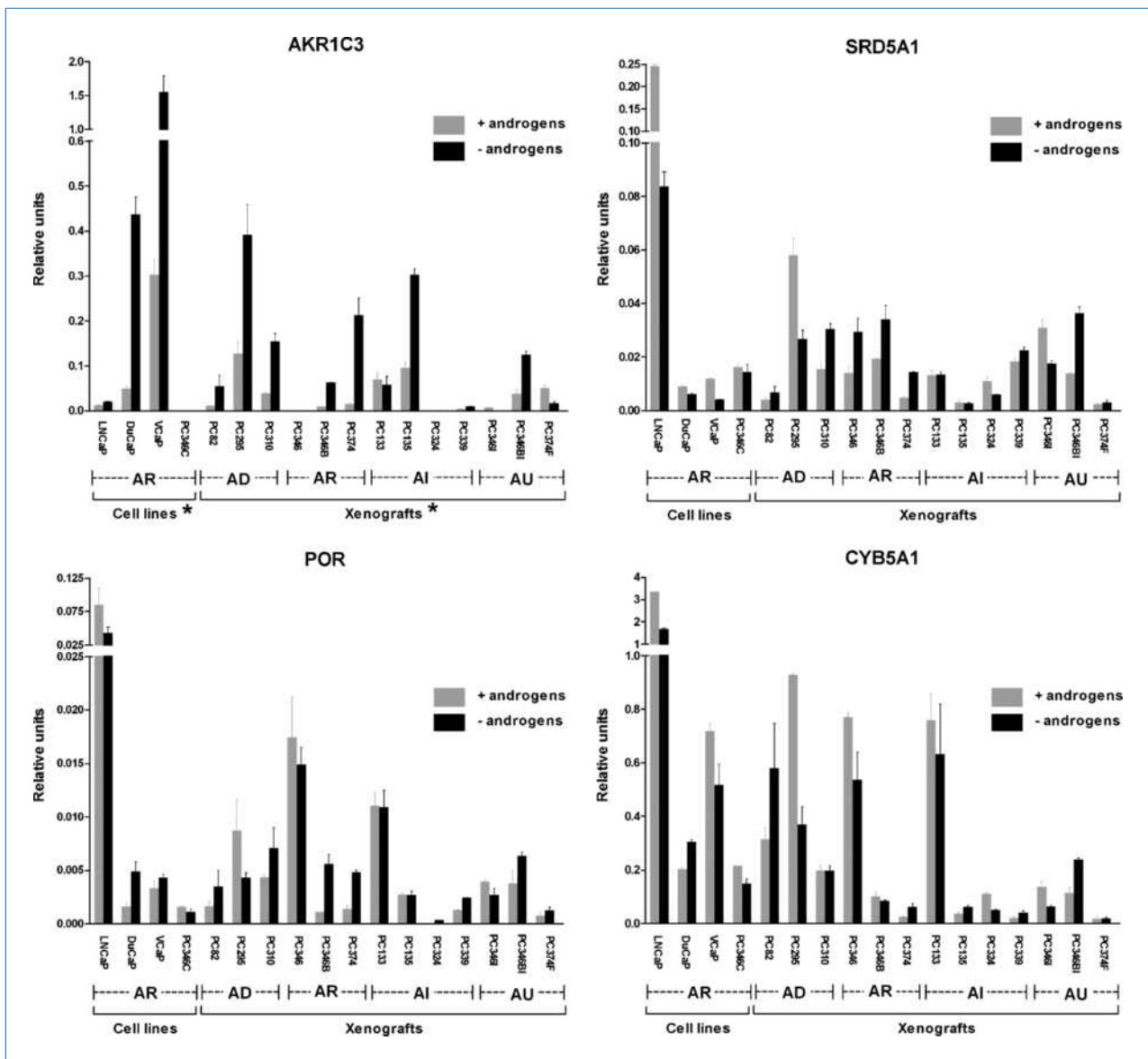

Figure 2. mRNA expression of the steroidogenic enzymes and coenzymes AKR1C3, SRD5A1, POR, and CYB5A1 in hormonally manipulated PC cell lines and xenografts. Four cell lines were treated with ethanol vehicle or 1 nmol/L R1881 for 8 h. Thirteen xenografts were obtained from control or castrated male nude mice. mRNA expression was calculated relative to the average of housekeeping genes *HPRT1* and *GAPDH. AD,* androgen dependent; *AR,* androgen responsive; *AI,* androgen independent; *AU,* androgen unresponsive. *, $P < 0.05$, relative values of treated versus control (paired *t* test). Data are presented as means and range of duplicates.
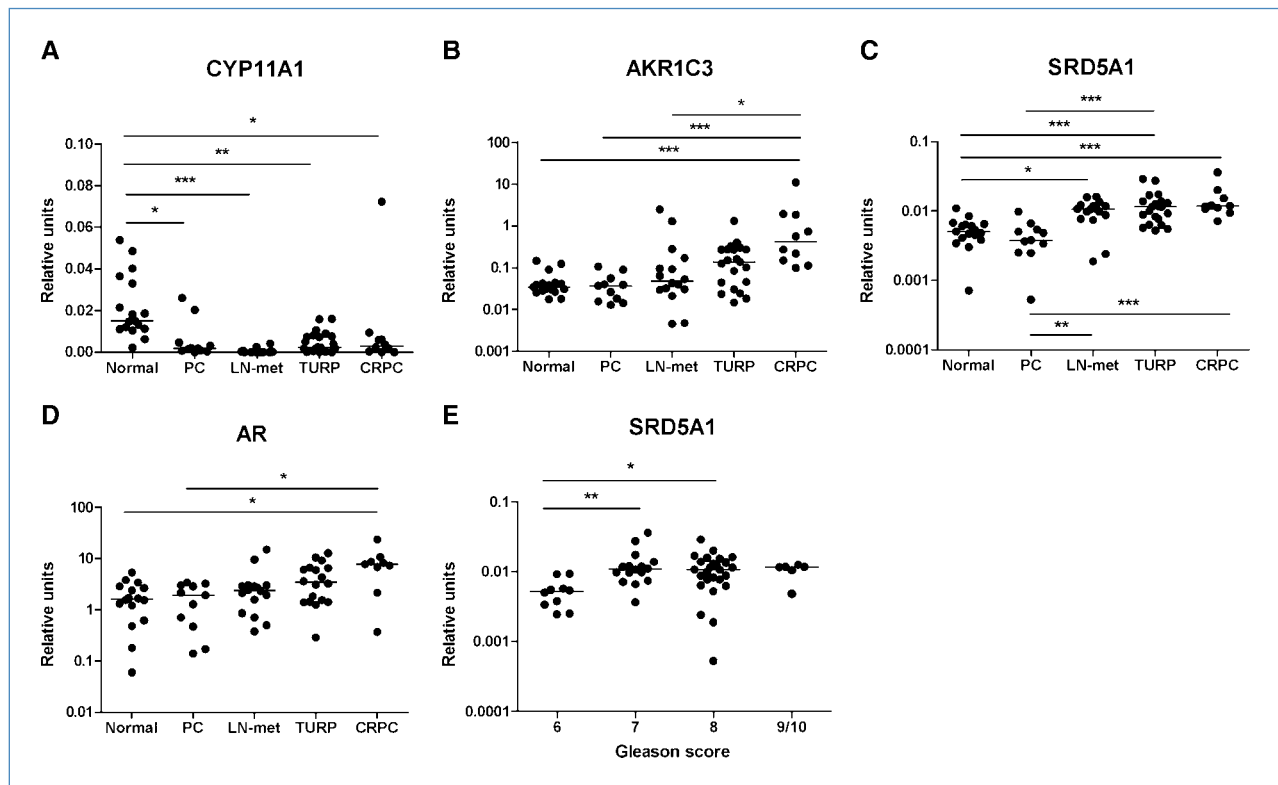
Figure 3. A to D, quantitative mRNA expression of the steroidogenic enzymes CYP11A1, AKR1C3, and SRD5A1 and the AR in normal prostate gland, local prostate carcinoma (*PC*), lymph node metastases (*LN-met*), locally advanced PC (*TURP*), and CRPC samples. E, association between SRD5A1 mRNA expression and Gleason score in the tumor samples. mRNA expression was calculated relative to the average of the housekeeping genes *HMBS* and *GAPDH*. Analyses were done using Kruskal-Wallis test and post hoc Dunn's multiple comparison test. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. Bars, means. Note: log scale in figures B to E.

Results

Cell lines and xenografts. The mRNA expression of STAR, CYP11A1, HSD3B1, HSD3B2, CYP17A1, POR, CYB5A1, AKR1C3, CYP19A1, SRD5A1, and SRD5A2 was measured in 4 different cell lines, which were treated with R1881 or vehicle, and in 13 different xenografts, grown in intact or castrated male mice. The absolute Ct values of these assays are shown in Supplementary Table S2. Quantitative reverse transcriptase-PCR (RT-PCR) for HSD3B2 displayed Ct values of ≥40 in all cell lines and xenografts studied. Analysis of the STAR, CYP11A1, HSD3B1, CYP17A1, CYP19A1, and SRD5A2 assays in all samples yielded Ct values in the higher ranges of 35.3 to ≥40, 30.8 to ≥40, 34.5 to ≥40, 34.1 to ≥40, 30.7 to ≥40, and 34.1 to ≥40, respectively, indicating very low expression. The expression of POR, CYB5A1, AKR1C3, and SRD5A1 was more pronounced, with Ct values in the range of 20 to 30 in all samples studied. The positive controls displayed appropriate Ct values in these assays, ranging from 18.7 to 27.0. In Fig. 2, the expression of the four above-mentioned mRNAs relative to housekeeping genes is shown. R1881 treatment suppressed AKR1C3 expression in all cell lines ($P = 0.022$). Intratumoral AKR1C3 expression was increased in castrated tumor-bearing mice as compared with tumors grown in intact mice ($P = 0.034$). R1881 treatment of cell lines or castra-

tion of tumor-bearing mice did not significantly alter the expression of SRD5A1. Hormonal manipulation also did not affect the mRNA expression of the ubiquitously present cofactors POR and CYB5A1 in these cell lines and xenografts.

Patient materials. We measured the expression of the steroidogenic enzymes CYP11A1, CYP17A1, HSD3B1, HSD3B2, AKR1C3, and SRD5A1 and the AR in the first series of 75 individual patient samples. Absolute Ct values are shown in Supplementary Table S3. CYP11A1 mRNA was detectable in 65 of 75 samples. When calculated relative to the housekeeping genes, the normal prostate tissue displayed higher expression levels of CYP11A1 than the four prostate carcinoma groups ($P < 0.05$; Fig. 3A). Ct values for HSD3B2 were again ≥40 in all samples studied. Expression of HSD3B1 and CYP17A1 varied, with Ct values in the range of 36.4 to ≥40 and 36.0 to ≥40, respectively. More importantly, all but four of the 75 patient samples did not show concomitant expression of CYP17A1 and HSD3B1, essential for *de novo* androstenedione and testosterone production. These levels were in the same range as found in other nonsteroidogenic tissues, such as liver, fat, and leucocytes (Supplementary Table S3). Of the proven CRPC samples, none was positive for both enzymes. Because of the importance of HSD3B1 and CYP17A1 for *de novo* production of androgens, we also measured their expression in the patient samples using commercially available certified RT-PCR assays

(Applied Biosystems). Results have been included in Supplementary Table S3 and Fig. 4. Again, the samples yielded high Ct values for HSD3B1 and CYP17A1, although these assays were slightly more sensitive than the previous sets (range, 32.9–≥40 and 31.5–≥40, respectively). No significant differences were found between the expression of HSD3B1 (Fig. 4A) or CYP17A1 (Fig. 4B) in the various groups of prostate tissues. Using these primer-probe sets, 13 of the 75 tissues, consisting of 5 normal prostate glands, 2 local PCs, 3 lymph node metastases, 2 TURP samples, and 1 CRPC sample, showed positive expression for both enzymes, all with Ct values in the range of 33.8 to 38.2 (Fig. 4C). In the additional TURP and CRPC tissues, very low expression of both HSD3B1 and CYP17A1 mRNAs was obtained as well (Supplementary Table S3). Relative expression of these samples has been added to Fig. 4A and B. Two locally advanced PC and four CRPC samples were positive for both HSD3B1 and CYP17A1 (Fig. 4C, *gray symbols*).

The mRNAs for AKR1C3 and SRD5A1 were detectable at substantially lower Ct values than obtained for HSD3B1 and CYP17A1, ranging from 24.2 to 33.6 and 28.2 to 36.0, respectively, in all samples tested in the first series. In Fig. 3B and C, the expression of these genes is indicated relative to that of housekeeping genes. AKR1C3 expression was significantly higher in CRPC samples compared with normal prostate,

local PC, and lymph node metastases. SRD5A1 mRNA was increased in lymph node metastases and both CRPC and non-CRPC TURP samples compared with normal prostate and local PC. SRD5A1 was associated with Gleason score: tissues with a Gleason score of 6 had lower SRD5A1 mRNA expression levels compared to tissues with higher Gleason scores (Fig. 3E). There was no association between AKR1C3 or SRD5A1 expression and the development of metastases or PSA progression during hormonal therapy. mRNA expression of the AR was detectable in all patient samples except for one normal prostate sample, three TURP samples, and one CRPC. When these samples were excluded from further analysis, AR expression was increased in CRPC compared with normal prostate and locally confined PC ($P < 0.05$; Fig. 3D). mRNA expressions of AR and AKR1C3 ($r = 0.387$; $P = 0.001$) were significantly correlated. AKR1C3 and SRD5A1 mRNA expressions were also correlated in these patient samples ($r = 0.486$; $P < 0.001$), but when corrected for AR expression, this relation was no longer significant (partial $r = 0.041$; $P = 0.869$).

Discussion

In this study, we quantified steroidogenic enzyme mRNA expression in a panel of experimental models of human PC
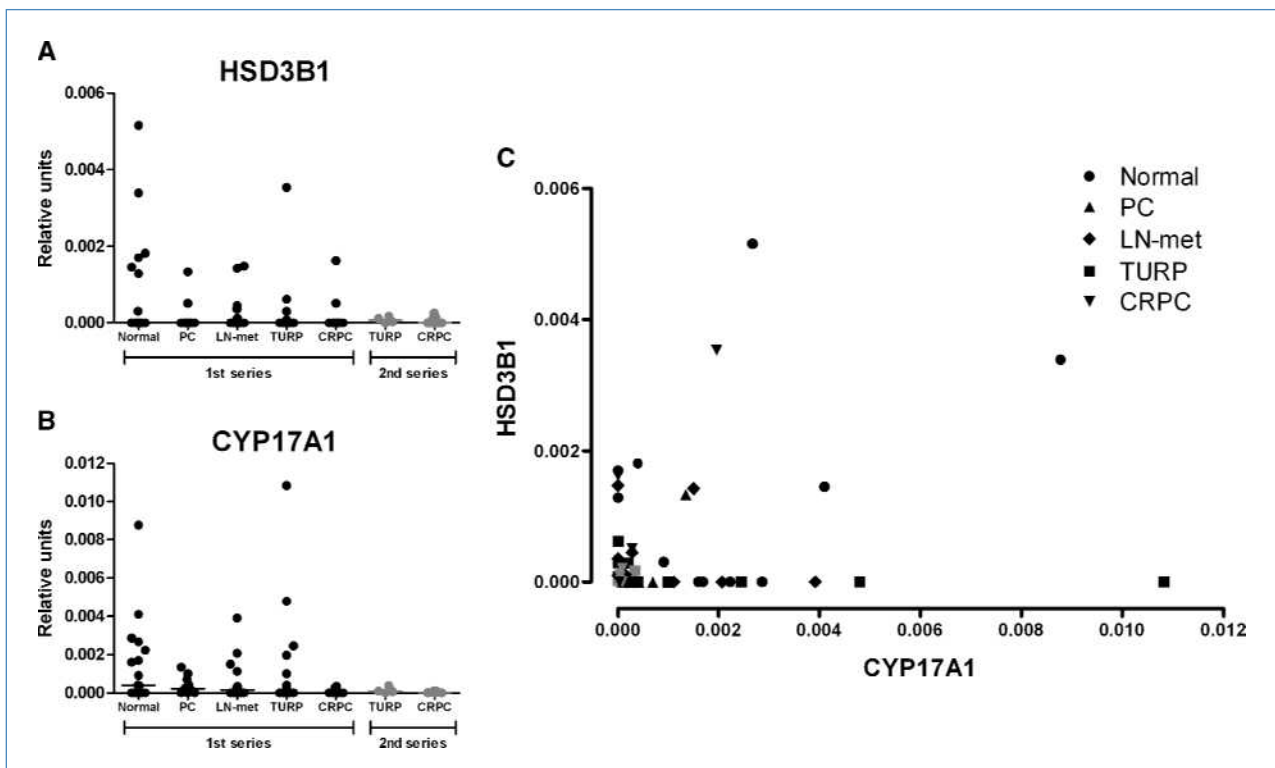

Figure 4. A and B, quantitative mRNA expression of HSD3B1 and CYP17A1 in the different types of samples using the commercially available assays. The second series only consisted of samples from locally advanced PC (TURP) or CRPC. These results have been depicted in gray. mRNA expression was calculated relative to the average of the housekeeping genes *HMBS* and *GAPDH*. *, $P < 0.05$. Bars, means. C, absence of concomitantly positive mRNA expression of CYP17A1 and HSD3B1 in the majority of human samples from normal prostate gland, local prostate carcinoma, lymph node metastases, locally advanced PC, and CRPC samples. *Black symbols,* first series of samples tested; *gray symbols,* second series. The 45 investigated tissues that displayed Ct values ≥40 for both enzymes are not depicted here. Moreover, HSD3B2 expression was absent in all samples.

and in human normal and tumorous prostatic tissues. In the majority of PC cases, simultaneous expression of all enzymes necessary for *de novo* synthesis of androgens from cholesterol to testosterone could not be detected. The enzymes that convert androstenedione to testosterone and testosterone to DHT were ubiquitously expressed and their levels were affected by endocrine therapy and related to the state of tumor progression.

In the experimental models for human PC, we detected Ct values in the range of ≥34 for STAR, CYP17A1, HSD3B1, HSD3B2, and SRD5A2, indicating very low mRNA copy numbers. Incubation with the synthetic androgen R1881 or castration of tumor-bearing mice did not alter the mRNA levels of these steroidogenic enzymes. In patient samples, we again investigated *de novo* androgen synthesis. Whereas HSD3B2 was negative in all samples, expressions of CYP17A1 and HSD3B1 excluded each other in the vast majority of samples, implying that enzymatic transformation of pregnenolone into androstenedione cannot occur in these tumor cells. Only in a subset of patients could levels of both enzymes be detected simultaneously albeit at low levels (Fig. 4C). These studies were conducted using a validated quantitative approach in samples that were evaluated for prostate tissue or tumor content, whereas positive controls for our assays (i.e., adrenal cortex, placenta, or normal prostate gland) showed readily detectable amplification of gene product. Detected Ct values were in the same range as in other nonsteroidogenic tissues. Moreover, in the patient samples, expression of the key enzymes HSD3B1 and CYP17A1 was tested with two different sets of primers and probes and confirmed in a second series of samples of locally advanced PC and CRPC. Overall, in this large series of experimental models and patient samples of PC, our findings plead for a limited role of intratumoral steroidogenesis.

We realize that our study is limited by the lack of distant metastatic PC tissues and that our conclusions are therefore limited to local PC, a limited number of CRPC, and lymph node metastases. Also, it should be realized that the quantitative mRNA levels determined in this study may not automatically reflect protein levels; modifications in (post-)transcriptional processes could still possibly lead to protein levels of the steroidogenic enzymes that might be sufficient for androgen production.

Recent studies on steroidogenic enzymes in PC showed evidence of the presence of STAR, HSD3B1, HSD3B2, and CYP17A1 expression in normal prostate or metastasized prostate carcinoma using immunohistochemistry, microarray, or quantitative RT-PCR (8, 9, 22). Importantly, these studies have only depicted relative expression values. The simultaneous expression of HSD3B1 and CYP17A1 in metastases as reported by Montgomery and colleagues (8) could only be confirmed in a limited number of TURP samples in our series, depending on the assay used. Possibly, this discrepancy is due to the fact that these authors investigated soft tissue metastases, in which upregulation or local expression could have played an important role. We have not studied those types of tissue, but also detected very low expression of HSD3B1 and CYP17A1 in normal prostate or

locally confined prostate carcinoma tissues. HSD3B1 expression may result from its presence in basal cells, which constitute a small population of the normal prostate (22). This could explain the higher percentage of positive expression in normal prostate samples compared with tumor tissues. Moreover, in contrast to Montgomery and colleagues (8), we used a probe-based assay with intron-spanning primers, which could have added to the specificity of our detection method.

Interestingly, CYP11A1 was detectable in the majority of normal prostate tissues, whereas its expression was strongly reduced in the tumor samples. Although this could lead to the formation of pregnenolone in these cells, pregnenolone itself does not possess biological activity and requires further metabolic transformation by CYP17A1. The expression of CYP11A1 could possibly be related to the CYP11A1-driven conversion of other substrates, such as 7-dehydrocholesterol and vitamin D3 (23). Vitamin D has been shown to exert effects on PC progression (24). Aromatase (CYP19A1) could only be located in a few xenografts, and castration resulted in a small increase of its expression (data not shown). This is in agreement with the observation by Hiramatsu and colleagues (25) that CYP19A1 immunoreactivity was only detectable in stromal cells adjacent to carcinoma cells.

The present results indicate that intraprostatic production of DHT could occur starting from androstenedione due to the presence of AKR1C3 and SRD5A1. The expression of AKR1C3 was negatively affected by androgens in both cell lines and xenografts. Furthermore, AKR1C3 expression was increased in CRPC compared with normal prostate, locally confined PC, and lymph node metastases, which is in line with previous immunohistochemical studies (11, 26) and microarray data (9). The upregulation of intratumoral AKR1C3 expression in patients receiving hormonal therapy, in addition to the presence of (over-)expressed AR, constitutes a plausible cause for the development of CRPC. The substrate for the AKR1C3-encoded enzyme would most likely be adrenal androstenedione, which is present in serum in the nanomolar range. *In vivo* studies using radiolabeled dehydroepiandrosterone (DHEA) and androstenedione have detected intraprostatic levels of labeled testosterone and DHT, although extraprostatic 3β-hydroxysteroid dehydrogenase activity cannot be ruled out in this setting (27). Steroid conversion into androgens has also been shown for androstenedione, DHEA, and DHEA-sulfate in isolated prostatic tissue (27, 28). The adrenocortical production of adrenal androgens therefore remains a crucial therapeutic target. Specific AKR1C3 inhibition for CRPC could also form a new therapeutic target: nonsteroidal anti-inflammatory drugs, selective cyclooxygenase-2 inhibitors, and steroid carboxylates have been proved to suppress this enzyme activity (29).

During tumor progression in prostate tissue, there is a switch of isoenzymes for 5α-reductase from SRD5A type II to SRD5A type I (9, 26, 30, 31). We could confirm these findings in our experimental models and found higher SRD5A1 levels in metastasized and hormone-refractory tumors. The association between SRD5A1 expression and Gleason score is in line with the observation that patients with a Gleason score of 7 to 10 PC had a smaller decline of intraprostatic

DHT concentrations during hormonal therapy compared with patients with a Gleason score of 6 or lower (32). However, it must be noted that in CRPC samples, the Gleason score is less reliable and could give an erroneously high value. The hypothesis that SRD5A1 is the main enzyme responsible for the local production of DHT in PC has important implications for the use of enzyme inhibitors in progressive disease. Based on these findings, treatment with finasteride, a selective type II 5α-reductase inhibitor, is likely to be less efficacious than treatment with an inhibitor of both types of enzymes, such as dutasteride (12).

Overall, we detected high Ct values for steroidogenic enzymes responsible for *de novo* androgen synthesis, indicating low copy numbers, whereas the enzymes responsible for the final conversions into testosterone and DHT were readily detected. The very high Ct values detected in our samples question the importance of these mRNAs in CRPC development. From our data, it is difficult to extrapolate whether the very low expression of STAR, HSD3B1, and CYP17A1 detected in our collection of PC samples is clinically relevant and also applies to distant metastatic lesions. Recently, Attard and colleagues (33) showed the efficacy of administration of the CYP17A1 inhibitor abiraterone acetate in patients with CRPC. The percentage of patients with PSA decline was comparable to that of similar patients receiving low-dose dexamethasone, which inhibits adrenal androgen production, in another study (34). This implies that the result of specific CYP17A1 inhibition is comparable to that of the blockade of adrenocortical steroid production and therefore suggests that intratumoral CYP17A1 plays a limited role in CRPC development compared with that of adrenal androgens. The limited role of intratumoral steroid conversion is further underlined by the absence of concomitant expression of HSD3B1 or HSD3B2 and CYP17A1 in 69 of the 88 patient samples, suggesting potential relevance only in a subset of patients. It seems most likely that the major source of intratumoral androgens after androgen deprivation therapy is blood-derived androstenedione, which, because of its nanomolar concentration, is an important substrate for the highly expressed enzymes AKR1C3 and SRD5A1, providing significant conversion into testosterone and DHT in the PC cells.

To study if intratumoral *de novo* steroid synthesis may still play a role in a subset of CRPC patients, adrenocortical blockade should first be administered. This setting can be mimicked in the *in vitro* models of cells grown in medium supplemented with charcoal-treated FCS and in the xenografts in castrated nude mice because the murine adrenal cortex is incapable of producing DHEA or androstenedione

due to its lack of CYP17A1 expression (35). Our study indicates that the expression of CYP17A1 and HSD3B1 is only detectable at very low levels in the androgen-responsive cell lines and two androgen-dependent xenografts. It must also be stated that through utilization of the recently discovered "backdoor pathway" of steroidogenesis (36), as was shown in small amounts for LNCaP cells (7), precursor 17-OH-progesterone could be converted into DHT in the samples in which CYP17A1 mRNA was detected. Although the increased SRD5A1 expression would be beneficial to this pathway, CYP17A1 levels are detected at a low range and HSD3B1 would still be necessary for *de novo* steroid synthesis.

We confirmed previous studies on AR expression in CRPC by showing expression of AR in the majority of samples (37, 38). The reported correlation of AR expression with expression of AKR1C3 (26) could also be confirmed. However, we could not replicate the previously reported correlation with SRD5A1 expression reported by the same authors.

In conclusion, enzymes for *de novo* synthesis of androgens are not highly expressed in the studied tissue samples of normal prostate gland, locally confined PC, lymph node metastases, and TURP from locally advanced PC and CRPCs, nor in experimental models of human PC. During tumor progression, SRD5A1 expression increases, whereas AKR1C3 expression increases during hormone ablation therapy, thus giving the prostate tumor an opportunity to convert circulating steroids of adrenal origin to testosterone and DHT locally and thereby to progress during hormonal therapy. Therefore, adjuvant treatment modalities should be directed to block adrenal androgen production. Additionally, the production of adrenal androgens as well as the putative presence of intratumoral *de novo* steroid biosynthesis in a subset of CRPCs may require additional inhibition of intratumoral AKR1C3 or SRD5A1 activity to disrupt the conversion of (adrenal) steroid precursors into active testosterone and DHT and, consequently, AR pathway activation.

Disclosure of Potential Conflicts of Interest

Acknowledgments

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