Steroidogenic Enzymes and Stem Cell Markers Are Upregulated during Androgen Deprivation in Prostate Cancer

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Considerable levels of testosterone and dihydrotestosterone (DHT) are found in prostate cancer (PCa) tissue after androgen deprivation therapy. Treatment of surviving cancer-initiating cells and the ability to metabolize steroids from precursors may be the keystones for the appearance of recurrent tumors. To study this hypothesis, we assessed the expression of several steroidogenic enzymes and stem cell markers in clinical PCa samples and cell cultures during androgen depletion. Gene expression profiles were determined by microarray or qRT-PCR. In addition, we measured cell viability and analyzed stem cell marker expression in DuCaP cells by immunocytochemistry. Seventy patient samples from different stages of PCa, and the PCa cell line DuCaP were included in this study. The androgen receptor (AR) and enzymes (AKR1C3, HSD17B2, HSD17B3, UGT2B15 and UGT2B17) that are involved in the metabolism of adrenal steroids were upregulated in castration resistant prostate cancer (CRPC). In vitro, some DuCaP cells survived androgen depletion, and eventually gave rise to a culture adapted to these conditions. During and after this transition, most of the steroidogenic enzymes were upregulated. These cells also are enriched with stem/progenitor cell markers cytokeratin 5 (CK5) and ATP-binding cassette sub-family G member 2 (ABCG2). Similarly, putative stem/progenitor cell markers CK5, c-kit, nestin, CD44, c-met, ALDH1A1, α2-integrin, CD133, ABCG2, CXCR4 and POU5F1 were upregulated in clinical CRPC. The upregulation of steroidogenic enzymes and stem cell markers in recurrent tumors suggests that cancer initiating cells can expand by adaptation to their T/DHT deprived environment. Therapies targeting the metabolism of adrenal steroids by the tumor may prove effective in preventing tumor regrowth.

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INTRODUCTION

As in normal prostate, the survival and growth of prostate cancer (PCa) cells also is driven by androgens. Therefore, androgen-deprivation therapy is the first-line treatment for clinically advanced PCa (1). Despite medical or surgical castration, in most of the patients, cancer recurs and may be considered as castration resistant prostate cancer (CRPC) (2). There are several proposed mechanisms leading to this incurable state of disease. Among others, the presence of cancer-initiating cells that are independent from androgens for their self-renewal, as well as the ability of PCa tissue to synthesize testosterone (T) and dihydrotestosterone (DHT) has been postulated (reviewed in 3).

Surgical and pharmacological androgen deprivation therapy aims to lower the amount of circulating testosterone. Secondary endocrine therapies are aimed to inhibit ligand binding to the androgen receptor (AR) with antiandrogens. However, several studies have confirmed that there are still sufficient amounts of T and DHT in the prostatic tissue to activate AR and its target genes that drive cancer cell survival and proliferation (4–8) even in the presence of the currently approved antiandrogens. Additionally, castration has little effect on the levels of adrenal androgens dehydroepiandrosterone (DHEA), its sulfate (DHEA-S) and androstenedione, by reducing them only 40% to 50% (4,7).

Androgen sensitivity of CRPC is demonstrated clearly by the fact that recurrent tumors respond to additional endocrine therapies (9,10). Several recent studies have reported upregulation of steroidogenic enzymes in CRPC which could lead to androgen production from adrenal androgens, and explain the levels of DHT and T found in prostate tissue (11–13). Moreover, there is evidence for de novo steroidogenesis from cholesterol by PCa cells (14). Supporting this observation, enzymes in the cholesterol biosynthesis pathway have shown to be upregulated in CRPC (15). Additionally, a number of studies provide evidence for stemlike cells that may be the initiating population for a recurrent tumor in PCa (16–20). Whether adaptation to low androgen levels is initiated by PCa stem
cells or by altering intracellular steroid metabolism is not yet known.

In this study, we compared the expression of steroidogenic enzymes in clinical samples of different stages of PCa and in the DuCaP cell line model. Many steroidogenic enzymes are upregulated in castration settings. In vitro these cells are enriched for some putative stem cell markers. Many stem cell markers are upregulated as well in clinical CRPC. This is the first evidence that links steroidogenesis with cancer-initiating cells in PCa.

MATERIALS AND METHODS

Patient Characteristics and Tissue Collection

Samples were collected at the Radboud University Nijmegen Medical Centre in 1987–2008, and the use was approved by the local ethical committee. On the basis of the pathology findings and the case records, patients were divided into four different groups: low-grade PCa (LG, n = 20), high-grade PCa (HG, n = 22), castration-resistant PCa (CRPC, n = 21) and metastatic PCa (met, n = 7) (Table 1). Castration-resistant status is defined as patients who failed conventional hormonal therapy. Upon radical prostatectomy, transurethral resection of the prostate or lymph node dissection, specimens were snap frozen in liquid nitrogen. Tissues were selected by microscopic assessment for purity of cancer cells and processed by step sectioning.

RNA Isolation

Total RNA was extracted from tissue sections by using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA was DNase treated and purified by using the RNaseasy Mini Kit (Qiagen, Venlo, the Netherlands) according to manufacturer’s instructions. Affymetrix GeneChip Whole Transcript Sense Target Labeling Assay (Affymetrix) was used to generate amplified and biotinylated sense-strand DNA targets from the entire expressed genome without bias. RNeasy purified total RNA (1 μg) was used, and an rRNA reduction step was included by using RiboMinus Human/Mouse Module (Invitrogen).

After hybridization by rotation at 0.33 g in the Affymetrix GeneChip hybridization oven at 45°C for 16 h, arrays were washed in Affymetrix GeneChip Fluidics station FS 450, and scanned by using Affymetrix Gene Chip scanner 3000 7G system (Affymetrix). Gene- and exon-level expression signal estimates were derived from CEL files generated from Affymetrix GeneChip Exon 1.0 ST arrays by using multiarray analysis algorithm implemented from Affymetrix Power Tools software (Affymetrix). Gene-level estimates were obtained by using the “core” metaprobe list annotation release 21.

Expression Analysis

Gene expression profiles were determined by using Affymetrix GeneChip 1.0 Human Exon ST (Affymetrix, Santa Clara, CA, USA) arrays according to manufacturer’s instructions. Affymetrix GeneChip Human Exon ST arrays by using multiarray analysis algorithm implemented from Affymetrix Power Tools software (Affymetrix). Gene expression in each sample was normalized with the housekeeping gene 2-microglobulin expression. Assays were performed three times.

Real-Time PCR

Two micrograms of Trizol-isolated total RNA was DNase treated, and cDNA was synthesized by using random primers (hexamers) and Superscript II Reverse Transcriptase (Invitrogen). Gene expression was determined by using SYBR Green PCR mix (Roche) and 10 ng of template. Real-time PCR was performed on a LightCycler LC480 instrument (Roche, Basel, Switzerland), using the following amplification conditions: 5 min at 95°C; followed by 50 cycles of 10 sec at 95°C, 20 sec at 60°C and 20 sec at 72°C. Cq-values were determined by using the LightCycler 480 SW 1.5 software (Roche). The primers were designed with OligoPerfect Designer (Invitrogen) or taken from a previous publication by Locke et al. (14) or Montgomery et al. (12). Primer sequences are listed in the Supplementary Table 1. Gene expression in each sample was normalized with the housekeeping gene (β2-microglobulin) expression. Assays were performed three times.

Cell Viability

Cells were seeded on 96-well plates (Corning Incorporated, Corning, NY, USA) 6,000 cells/well in RPMI-1640 supplemented with 10% FCS or CSS. Half of the medium was changed every fourth day. A standard MTT

Table 1. Patient characteristics.

| Group   | LG | HG | met | CRPC |
|---------|----|----|-----|------|
| Number of patients | 20 | 22 | 7   | 21   |
| Previous treatment | none | none | none | surgical or medical castration with or without antiandrogens |
| Site of metastasis | N/A | N/A | LN (n = 6) tests (n = 1) | N/A |
| Gleason score | 4 (n = 6) | 7 (n = 3) | 5 (n = 4) | 9 (n = 3) |
|                     | 6 (n = 10) | 8 (n = 11) | 10 (n = 5) | 8 (n = 11) |

N/A, not applicable; LN, lymph node.
(Methylthiazolyl diphenyl-Tetrazolium bromide, Sigma-Aldrich) assay was performed up to 24 d. Experiments were repeated three times with triplicates.

**Immunocytochemistry**

Cells were grown for 7 d on glass slides or on collagen I coated slides for α2-integrin staining, and fixed with ice cold acetone for 10 min or with 4% paraformaldehyde at RT for 10 min. Acetone-fixed cells were stained with monoclonal antibodies; CK18 (clone DC10, DAKO), cytokeratin 5 (CK5) (clone RCK103, Euro-Diagnostica, Malmö, Sweden), BCRP (Millipore, Billerica, MA, USA), CD49b (α2-integrin, clone Gi-14; from S Santoso, Giessen, Germany), and with the polyclonal antibody c-kit (A4502, Dako, Glostrup, Denmark). The 4% paraformaldehyde-fixed cells were stained with CD133/2 (Miltenyi Biotec, Bergisch Gladbach, Germany) and nestin (Millipore). CK18 (1:100), CK5 (1:2),
CD49b (1:400), c-kit (1:200), and CD133/2 (1:200 dilution) were incubated for 1–2 h at RT and antibodies against nestin (1:200) and BCRP (1:100) overnight at 4°C. Powervision poly-HRP-goat antimouse/rabbit/rat IgG (ImmunoLogic, Duiven, the Netherlands) was used as the secondary antibody. Except in the case of c-kit, where goat-an-tirabbit secondary antibody RPN1004V (1:200, GE Healthcare, Buckinghamshire, UK) was used with subsequent incubation with Vectastain ABC-peroxidase solution (Vector, Burlingame, CA, USA). Detection of antibody binding was performed by using 3,3′-diaminobenzidine (Power DAB, ImmunoLogic) to observe peroxidase activity. The nuclei were counterstained with hematoxylin.

Known positive tissues or cells were used as controls. The staining was studied under a light microscope. All positive cells on slides were counted, and the number of negative cells was estimated by multiplying the counted cells on representative fields by the slide area.

All supplementary materials are available online at www.molmed.org.

RESULTS

Expression of Steroidogenic Enzymes Is Altered in Clinical CRPC

The mRNA expression of genes involved in steroid metabolism (Figure 1) and androgen signaling in patient samples of LG, HG, met and CRPC were studied by microarray analysis. Most of the statistically significant differences in gene expression appeared in CRPC (Table 2). Expression values with the most differences are shown as box plots in Figure 2. The expression of AR and steroidogenic enzymes AKR1C3, HSD17B2, HSD17B3, UGT2B15 and UGT2B17, was generally upregulated in CRPC, whereas SRD5A2 was downregulated. AKR1C3 expression was significantly different in CRPC versus LG (P = 0.001), and for HSD17B3 in met versus LG (P = 0.049) and HG (P = 0.016), and CRPC versus LG (P = 0.004) and HG (P = 0.024) were significantly different. SRD5A2 expression was different in met versus LG and HG, and CRPC compared with LG, HG and met (P = 0.000, 0.000, 0.000, 0.000 and 0.003, respectively). UGT2B15 expression was different between CRPC and LG (P = 0.001), and HG (P = 0.032) and met (P = 0.001). UGT2B17 was upregulated in CRPC versus LG (P = 0.001) and HG (P = 0.023). In addition, the expression of AR was significantly different in met versus LG, and in CRPC versus LG, HG and met (P = 0.000, 0.000, 0.000 and 0.044, respectively).

Expression of Steroidogenic Enzymes Is Altered in DuCaP during Hormone Depletion

To examine the expression of steroidogenic enzymes during short-term androgen ablation, we cultured DuCaP cells in medium containing CSS for 24, 48, 96 h and 12 d. These conditions also were maintained for several passages to study the long-term effect of androgen ablation. Only a small

Table 2. Mean fold-change values in mRNA expression of steroid metabolism/signaling related genes in clinical samples of LG, HG, met and CRPC.

| Gene          | Gene bank accession number | HG versus LG | met versus LG | met versus HG | CRPC versus LG | CRPC versus HG | CRPC versus met |
|---------------|-----------------------------|--------------|---------------|---------------|----------------|----------------|-----------------|
| FASN          | NM_004104                   | 0.8          | 1.1           | 1.3           | 1.0            | 1.2            | 0.9             |
| STAR          | NM_000349                   | 1.0          | 1.5           | 1.5           | 1.1            | 1.1            | 0.7             |
| CYP11A1       | NM_001099773                | 1.0          | 1.2           | 1.2           | 1.0            | 1.0            | 0.9             |
| HSD3B2        | BC131488                    | 1.0          | 1.0           | 1.0           | 1.1            | 1.1            | 1.0             |
| HSD17B2       | NM_002153                   | 1.0          | 1.0           | 1.0           | 1.1            | 1.0            | 1.0             |
| HSD17B3       | NM_000197                   | 1.1          | 1.3(a)        | 1.2(a)        | 1.3(b)         | 1.3(b)         | 1.0             |
| HSD17B4       | NM_000414                   | 1.0          | 0.8           | 0.8           | 0.8            | 0.8            | 1.0             |
| HSD17B10      | NM_004493                   | 1.0          | 1.1           | 1.1           | 1.0            | 1.0            | 0.9             |
| AKR1C1        | NM_001353                   | 2.2          | 1.3           | 0.6           | 2.5(b)         | 1.5            | 1.4             |
| AKR1C2        | NM_00205845                 | 1.2          | 1.1           | 0.9           | 1.0            | 0.8            | 0.9             |
| AKR1C3        | NM_003739                   | 1.5          | 1.5           | 1.0           | 2.3(b)         | 1.5            | 1.4             |
| RDH5          | NM_002905                   | 1.0          | 1.1           | 1.1           | 1.1            | 1.1            | 1.1             |
| SRD5A1        | NM_001047                   | 1.0          | 1.0           | 1.0           | 1.1            | 1.1            | 1.0             |
| SRD5A2        | NM_003348                   | 0.8          | 0.2(b)        | 0.2(b)        | 0.3(b)         | 0.4(b)         | 1.8(b)          |
| SRD5A3        | NM_0024592                  | 0.9          | 1.1           | 1.1           | 1.0            | 1.1            | 1.0             |
| UGT2B15       | NM_001076                   | 1.5          | 1.4           | 0.9           | 2.5(b)         | 1.7(a)         | 1.8(b)          |
| UGT2B17       | NM_001077                   | 1.2          | 1.3           | 1.0           | 1.7(b)         | 1.4(b)         | 1.3             |
| AMACR         | NM_014324                   | 1.2          | 0.8           | 0.6           | 0.6(a)         | 0.5            | 0.7             |
| SREB1         | NM_00005291                 | 0.9          | 1.0           | 1.1           | 0.8(a)         | 0.8            | 0.8             |
| SREB2         | NM_0004599                  | 0.9          | 1.4(a)        | 1.5(b)        | 1.0            | 1.0            | 0.7(b)          |
| CYB5A         | NM_14823                    | 0.8(a)       | 0.7(b)        | 0.9           | 0.6(b)         | 0.7            | 0.9             |
| AR            | NM_0000446                  | 1.0          | 1.7(b)        | 1.6           | 4.1(b)         | 4.0(b)         | 2.5(b)          |
| ERG           | NM_0004449                  | 0.7          | 1.6           | 2.1           | 0.7            | 1.0            | 0.5             |
| KLK3          | NM_001030047                | 0.7          | 0.9           | 1.3           | 0.6(b)         | 0.9            | 0.7             |
| TMPRSS2       | NM_005656                   | 0.5(a)       | 0.5           | 1.0           | 0.8(b)         | 1.6            | 1.6             |

FASN, fatty acid synthase; STAR, steroidogenic acute regulatory protein; CYP, cytochrome P450; HSD, hydroxysteroid dehydrogenase; AKR, aldo-keto reductase; RDH, retinol dehydrogenase (oxidative 3′-HSD); SRD, 5α-reductase; UGT, UDP glucuronosyltransferase; AMACR, α-methylacyl-CoA racemase; SREB, G protein-coupled receptor; CYB5, cytochrome b5; ERG, v-ets erythroblastosis virus E26 oncogene homolog; KLK, kallikrein-related peptidase; TMPRSS2, transmembrane protease, serine 2.

Statistical analysis: t test. P < 0.05.

Statistical analysis: t test. P < 0.01.
subset of cells survived. Finally, within 5 months, a culture adapted to hormone deficient environment was proliferating, and could be maintained in these conditions. The mRNA expression of genes involved in steroid metabolism is upregulated in hormone depleted conditions both short term (Figure 3) and long term (Figure 4). Only HSD17B2 was downregulated in long-term hormone depletion.

**Hormone Depletion Surviving Cells Can Repopulate the Culture after Hormonal Restoration**

Only a small population of DuCaP cells remained viable during 24 d of hormonal depletion, but this population did not increase in number during the experiment. However, when normal hormonal conditions were restored, these cells repopulated the culture (Figure 5).

**Hormone Depletion Surviving Cells Express Putative Stem/Progenitor Cell Markers**

The expression of putative stem/progenitor cell markers was assessed by immunocytochemistry in DuCaP. The number of CK5 and ATP-binding cassette sub-family G member 2 (ABCG2)-expressing cells in hormone-depleted conditions was 1.3-fold and 14.9-fold higher, respectively, than in normal conditions. Owing to high standard deviation between experiments, we could not show statistical significance in the measurements. Most cells in both culture conditions were positive for α2-integrin. No staining was detected for c-Kit, nestin or CD133 (Table 3 and Figure 6).

**Expression of Putative Stem/Progenitor Cell Markers Is Upregulated in Clinical CRPC**

We studied the mRNA expression levels of several putative stem/progenitor cell markers in patient samples from metastatic PCA and CRPC by microarray. These samples of pure cancer tissue were chosen to avoid the effect of possible presence of normal cells on the expression values. CK5, c-Kit, nestin, CD44 and...
c-Met were upregulated significantly in CRPC compared with noncastrated clinical metastatic disease \( (P = 0.000, 0.007, 0.000, 0.036 \text{ and } 0.018, \text{ respectively}) \). A trend in higher expression of putative stem cell markers aldehyde dehydrogenase 1 family, member A1 \((\text{ALDH1A1})\), \(\alpha\)2-integrin, CD133, ABCG2 and C-X-C chemokine receptor type 4 \((\text{CXCR4})\), both POU class 5 homeobox 1 \((\text{POU5F1}; \text{alternative name Oct3/4})\) transcript variants, and lower expression of \(\alpha\)6-integrin, SRY (sex determining region Y)-box 2 \((\text{SOX2})\) and Nanog homeobox \((\text{Nanog})\) was noticed in CRPC, but without statistical significance. (Table 4 and Figure 7).

**DISCUSSION**

Similar to recent studies \((11–14)\), we show that several steroidogenic enzymes are upregulated in CRPC. In particular, the gene expression of the enzymes \((\text{HSD17B2, HSD17B3, AKR1C3, SRD5A2, UGT2B15 \text{ and } UGT2B17})\) of the last steps in the steroidogenic pathway from adrenal androgens to T, DHT and their elimination to glucuronide derivatives were changed. Compared to Montgomery \textit{et al}. \((12)\) and Hofland \textit{et al}. \((11)\), we could not detect a significant upregulation of \(\text{SRD5A1}\) in CRPC. However, the expression level of \(\text{SRD5A2}\) was downregulated, resulting in a possible takeover by \(\text{SRD5A1}\) as the main 5a-reductase. The activation of the steroid metabolism simultaneous toward and away from the active steroids clearly indicates that this balance is controlled carefully in the tissue. Like presented by Hofland \textit{et al}. \((11)\), the last steps in steroidogenesis seem pivotal in the adaptation to castrate state. Therapies targeting these steps of steroidogenesis may prove to be an important addition to current endocrine therapies.

DuCaP is a representative model to study CRPC, since it expresses wild type AR, possesses \(\text{TMPRSS2-ERG}\) translocation and contains stromal cells. We have shown previously that endocrine therapy can be mimicked \textit{in vitro} by growing DuCaP cells in CSS. \((21)\) In these conditions, all tested steroidogenic enzymes are upregulated and become more pronounced in time. Despite the changes in the steroidogenic pathway, the cells in hormone-depleted conditions do not proliferate. However, after being subjected for a long period of time to poor hormonal environment, a new cell culture arose adjusted to these conditions. Previ-
DuCaP was suggested to contain stemlike cells (19). Since only a small cell population survives androgen depletion in vitro, and is capable to repopulate the culture, these surviving cells may indeed be stemlike. Immunostainings for several stem/progenitor cell markers revealed an increase in CK5- and ABCG2-expressing cells. This observation was extended to clinical PCa; the gene expression of many stem cell markers was upregulated in CRPC compared with nontreated PCa. Immunohistochemical studies in clinical samples are needed to further validate the results by us and others, as investigation on mRNA does not necessarily reflect the expression level of the protein in question. We are currently studying AKR1C3 in CRPC in more depth. Additionally, studies on hormone levels in DuCaP would reveal more functional information about the steroidogenetic enzymes as demonstrated by Locke et al. (14). We analyzed testosterone and DHT levels in DuCaP-conditioned hormone-depleted medium by liquid chromatography/radioimmunnoassay. Unfortunately with this method the detection level threshold was too low (T: 0.02 and DHT: 0.1 nmol/L) to detect any change in metabolism in this type of samples.

We approached the problem of PCa recurrence after therapy with a novel point of view by the combination of stemlike cells and steroid metabolism. The upregulation of steroidogenetic enzymes and stem cell markers in recurrent tumors suggests a linkage between steroidogenesis and stem cells. As the endocrine therapy targets the androgen sensitive cells of the tumor, stemlike cells survive the deprivation. These cells may give rise to cells that have adapted their androgen metabolism. The remaining adrenal androgens may then be utilized to restore the appropriate androgen environment for the recurrent tumor.

Taken together, enzymes involved in steroidogenesis are upregulated both in clinical CRPC and in castration settings.

**Figure 6.** Immunohistochemical stainings for putative stem/progenitor cell markers in DuCaP. Cells were grown in RPMI-1640 supplemented with 10% FCS or 10% CSS for 7 d and stained for putative stem/progenitor cell markers. Positive staining was found for CK5, ABCG2 and α2-integrin. No staining was detected for c-Kit, nestin or CD133. Known positive tissues or cells were used as controls. Scale: 0.2 mm.

**Table 4.** Mean fold-change values in mRNA expression of putative stem cell markers in clinical samples.

| Gene                  | Gene bank accession number | CRPC versus met |
|-----------------------|----------------------------|-----------------|
| CK5                   | NM_000424                  | 3.9a            |
| c-Kit                 | NM_000222                  | 3.3a            |
| Nestin                | NM_006617                  | 1.5a            |
| CD44                  | NM_000610                  | 2.2b            |
| c-Met                 | NM_000245                  | 2.0b            |
| ALDH1A1               | NM_00689                   | 1.7             |
| α2-Integrin           | NM_002203                  | 1.6             |
| CD133                 | NM_006017                  | 1.6             |
| ABCG2                 | NM_004827                  | 1.4             |
| CXCR4                 | NM_01008540                | 1.3             |
| POU5F1 variant 1      | NM_002701.4                | 1.1             |
| POU5F1 variant 2      | NM_203289.4                | 1.1             |
| α6-Integrin           | NM_000210                  | 0.8             |
| SOX2                  | NM_003106                  | 0.8             |
| Nanog                 | NM_024865                  | 0.5             |

*aStatistical analysis: t test, P < 0.01.
*bStatistical analysis: t test, P < 0.05.
*cCD133, prominin 1.

**Figure 7.** mRNA expression of putative stem cell markers in clinical samples of met and CRPC. Analysis was done by using the t test. Horizontal line, median value; open circle, outliers; asterisk, extremes. CD133, prominin 1.
in vitro. Also, an enrichment of stem cell markers was shown in these samples. Current endocrine therapies may induce a selection for stemlike cancer cells and an induced steroidogenesis. Our results support the rationale to combine castration and antiandrogen treatment with inhibitors that block adrenal androgen metabolism and autocrine steroidogenesis in prostate tissue. This may prove to be a more successful approach to preventing disease progression to CRPC.

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