Differential expression of steroid 5α-reductase isozymes and association with disease severity and angiogenic genes predict their biological role in prostate cancer

Kakoli Das1,2, Pia D N Lorena2, Lai Kuan Ng2, Diana Lim2, Liang Shen3, Woei Yun Siow4, Ming Teh2, Juergen K V Reichardt5 and Manuel Salto-Tellez1,2

1Cancer Science Institute, 2Department of Pathology, 3Biostatistics Unit and 4Department of Urology, National University Health System, National University of Singapore, 28 Medical Drive, Singapore, Singapore 117456
5Plunkett Chair of Molecular Biology (Medicine), University of Sydney, Sydney, New South Wales 2006, Australia

(Correspondence should be addressed to K Das at Cancer Science Institute, National University of Singapore; Email: kaks23@yahoo.com; M Salto-Tellez at Cancer Science Institute, National University of Singapore; Email: patmst@nus.edu.sg)

Abstract

The biological role of steroid 5α-reductase isozymes (encoded by the SRD5A1 and SRD5A2 genes) and angiogenic factors that play important roles in the pathogenesis and vascularization of prostate cancer (PC) is poorly understood. The sub-cellular expression of these isozymes and vascular endothelial growth factor (VEGF) in PC tissue microarrays (n = 62) was examined using immunohistochemistry. The effect of SRD5A inhibition on the angiogenesis pathway genes in PC was also examined in prostate cell lines, LNCaP, PC3, and RWPE-1, by treating them with the SRD5A inhibitors finasteride and dutasteride, followed by western blot, quantitative PCR, and ELISA chip array techniques. In PC tissues, nuclear SRD5A1 expression was strongly associated with higher cancer Gleason scores (P = 0.02), higher cancer stage (P = 0.01), and higher serum prostate specific antigen (PSA) levels (P = 0.01), whereas nuclear SRD5A2 expression was correlated with VEGF expression (P = 0.01). Prostate tumor cell viability was significantly reduced in dutasteride-treated PC3 and RWPE-1 cells compared with finasteride-treated groups. Expression of the angiogenesis pathway genes transforming growth factor β 1 (TGFβ1), endothelin (EDN1), TGFα (TGFA), and VEGFR1 was upregulated in LNCaP cells, and at least 7 out of 21 genes were upregulated in PC3 cells treated with finasteride (25 µM). Our findings suggest that SRD5A1 expression predominates in advanced PC, and that inhibition of SRD5A1 and SRD5A2 together was more effective in reducing cell numbers than inhibition of SRD5A2 alone. However, these inhibitors did not show any significant difference in prostate cell angiogenic response. Interestingly, some angiogenic genes remained activated after treatment, possibly due to the duration of treatment and tumor resistance to inhibitors.

Endocrine-Related Cancer (2010) 17 757–770

Introduction

Prostatic carcinogenesis and growth are largely dependent on the vital male hormone dihydrotestosterone (DHT), the most bioactive androgen, and the process of neovascularization called angiogenesis. Production of androgens in prostate cells occurs by the action of two isozymes, steroid 5α-reductase types I and II, encoded by the SRD5A1 and SRD5A2 genes respectively (Bruchovsky et al. 1988, Bonkhoff et al. 1996). Angiogenesis is regulated by several stimulating and inhibiting growth factors, the most potent stimulatory factor being vascular endothelial growth factor (VEGF; Leung et al. 1989, Boddy et al. 2005). Recently, a third SRD5A, encoded by the
SRD5A3 gene, has been suggested to be associated with DHT production and androgen receptor (AR) activation in hormone refractory prostate cancer (PC; Uemura et al. 2008). Its role, however, remains obscure. SRD5A1 and SRD5A2 are membrane-associated enzymes, composed of 259 and 254 amino acids respectively. The genes encoding them, SRD5A1 and SRD5A2, map to chromosome bands 5p15 and 2p23 respectively, and are mainly expressed in the liver, skin (Thigpen et al. 1993, Russell & Wilson 1994), and PC tumors (Thigpen & Russell 1992, Reichardt et al. 1995, Nakamura et al. 2005). Polymorphisms in the SRD5A2 gene were previously demonstrated to be associated with PC susceptibility (Makridakis et al. 1999), although the same group recently revisited their multi-ethnic cohort data and reported no association between the alanine-49 to threonine (A49T) missense variant of the SRD5A2 gene and PC risk (Pearce et al. 2008). We also studied the valine-89 replaced by leucine (V89L) single nucleotide polymorphism of the SRD5A2 gene reported in a Singapore hospital. However, further study failed to find any such correlation between this variant and PC risk (Das et al. 2008a).

Both SRD5A isozymes are thought to be important in the local production and action of DHT in normal and neoplastic prostatic tissue. Cellular interactions in the prostate allow 5α-reductase to metabolize testosterone to DHT, which forms a complex with AR, which is then translocated to the nucleus. In contrast, androgen target cells in peripheral tissues use testosterone directly to activate ARs. The DHT–AR complex then binds to nuclear DNA sequences known as androgen response elements, which promote transcription of the target genes involved in prostate cell homeostasis, angiogenesis, differentiation, and apoptosis (Russell & Wilson 1994, Gelmann 2002). Testosterone activates AR in the same manner as DHT. However, DHT dissociates from AR more slowly than testosterone (Russell & Wilson 1994); hence, most ARs in the prostate are occupied by DHT under steady conditions. A major role of 5α-reductase, therefore, is to enhance the androgen effect by allowing tighter binding to the AR.

The expression level of SRD5A is elevated in benign prostatic hyperplasia (BPH) and PC, resulting in enhanced production of DHT and overexpression of AR (Thomas et al. 2003, 2005, Nakamura et al. 2005, Wako et al. 2008). However, the biological role of the two 5α-reductase isoforms in PC and BPH is not clearly understood. Some studies have shown a switch from SRD5A2 to SRD5A1 expression in recurrent PC (Titus et al. 2005) and loss of expression of both isoforms in metastatic PC (Habib et al. 2003). Others, however, have shown higher expression levels of SRD5A1 and SRD5A2 in both types of PC (Nakamura et al. 2005), and enhanced expression of SRD5A1 in PC compared with BPH tissues (Bonkoff et al. 1996, Thomas et al. 2003, Wako et al. 2008).

Prostate carcinogenesis is a slow, stepwise process, and medical approaches are necessary to either delay the natural history of the disease or prevent it altogether. The 5α-reductases offer a mechanism for reducing androgen stimulation of the prostate without lowering circulating testosterone levels (Rittmaster et al. 2009). Although SRD5A2 is known to be the predominant gene in the prostate and a prominent therapeutic target for BPH, its potential as a therapeutic agent for PC is currently unknown. Finasteride, an SRD5A2 inhibitor that prevents the conversion of testosterone to DHT, is a widely used drug for the treatment of BPH. It is also used for BPH-related hematuria, since expression of VEGF and microvessel density are affected by SRD5A2 activity (Pareek et al. 2003). Finasteride has been shown to stimulate VEGF expression in other cells as well, e.g. dermal papilla cells, which are targets for many circulating hormonal and growth factors (Lachgar et al. 1999). Dutasteride, another inhibitor of 5α-reductase, blocks the activity of both isoforms, and is currently being investigated for both PC risk reduction and treatment (Rittmaster et al. 2009). Higher androgen levels, as noted in primary and metastatic PC, increase VEGF and AR expression (Joseph et al. 1997), but have the opposite effect in androgen-deprived PC (Cheng et al. 2004, Aslan et al. 2005). However, whether or not VEGF, or other angiogenic genes, is regulated by 5α-reductase remains uncertain.

In order to understand the relationship between SRD5A1 and SRD5A2 and their effects on the PC cell angiogenic response, we investigated the expression of SRD5A1 and SRD5A2, AR, and the key angiogenic molecule VEGF in PC tissue microarrays (TMAs), and correlated them with clinico-pathological characteristics of PC patients. Furthermore, expression of a panel of genes in the angiogenesis pathway was also examined in selected tissues by quantitative PCR. Malignant and non-malignant prostate cells were also treated with finasteride and dutasteride in order to analyze the expression of these angiogenesis pathway genes. Our results showed an increase in SRD5A1 expression in PC tissues, which was correlated with disease aggressiveness and upregulation of certain genes in the angiogenesis pathway in response to both finasteride and dutasteride, despite a reduction in tumor cell viability.
**Materials and methods**

**Patient tissues**

PC and matched benign tissues were consecutively obtained from 62 patients who had undergone radical prostatectomy \((n = 25)\) and transurethral prostatectomy \((n = 37)\) without prior treatments such as hormone therapy, chemotherapy, or radiotherapy at the Division of Surgery, National University Hospital (NUH), Singapore, between 2000 and 2007. All prostate specimens were acquired in compliance with the guidelines of the Institutional Review Board and ethics committee including patients’ informed consent (DSRB-B/09/141). Table 1 shows the characteristics of the patients included in the study. Histopathological staging of PC was based on the 1997 TNM-staging system (Sobin & Fleming 1997). Patients were followed from the time of diagnosis until death. Alternatively, those patients with a minimum of 3 years of followup were included for the survival analysis.

**Immunohistochemistry**

Immunohistochemistry was performed on TMA constructed from formalin-fixed paraffin-embedded (FFPE) prostate tumors and matched benign tissues following the protocol described previously (Zhang et al. 2003, Salto-Tellez et al. 2007, Das et al. 2008b). Sections \((4 \mu m)\) were deparaffinized and rehydrated using graded alcohols before heating with antigen retrieval solution \((pH 6)\) at 120°C for 5 min, followed by peroxidase blocking for 10 min at room temperature. Primary polyclonal antibodies for human VEGF \((\text{dilution } 1:20)\) (sc-152, Santa Cruz Biotechnology, Santa Cruz, CA, USA), AR \((\text{dilution } 1:1500)\) (Chemicon International, Temecula, CA, USA), and SRD5A1 \((\text{dilution } 1:100)\), and monoclonal antibodies for SRD5A2 \((\text{dilution } 1:50)\) (Abnova Corporation, Taipei City, Taiwan) were used, followed by incubation with biotinylated anti-mouse/rabbit secondary HRP-conjugated streptavidin (DAKO Real, Cambridgeshire, UK). Peroxidase activity was then visualized using diaminobenzidine substrate. As a negative control, normal rabbit or mouse IgG was used instead of the primary antibodies, with no specific immunoreactivity observed in these tissue sections.

TMA-immunostained sections were visually scored by two observers (P D and K D) who were blinded to the clinical outcome of the patients. Scoring was then confirmed by an experienced pathologist (M S T). Within each tissue sample, expression in the tumor cells was relatively homogenous. The staining was semi-quantitatively assessed on a scale ranging from 0 to 3, termed as ‘expression scores’ \((0, \text{no expression}; 1, \text{weak expression}; 2, \text{moderate expression}; 3, \text{strong expression})\), following our previously published protocols (Zhang et al. 2003). To determine the protein expression correlation between BPH and PC by immunohistochemistry, expression scores 0 and 1 were grouped together and assessed as ‘negative expression’, and expression scores 2 and 3 were grouped together and assessed as ‘positive expression’, as described previously (Das et al. 2009). Tumors that could not be scored due to insufficient tissue cores in the TMA were discarded from the study.

**Cell culture**

Two established human PC cell lines, LNCaP and PC3 (ATCC, Manassas, VA, USA), were maintained in RPMI 1640 and F-12 media (Gibco), supplemented with 10% heat-inactivated fetal bovine serum and 1% L-glutamine respectively. Another prostate cell line derived from normal prostate cells, RWPE-1 (ATCC), was maintained in keratinocyte serum-free medium (Gibco) supplemented with 0.05 mg/ml bovine pituitary extract and 5 ng/ml epidermal growth factor. Cell lines were seeded in 12-well plates at a density of \(1 \times 10^5\) cells/ml and incubated for 24 h. They were then treated with finasteride (Sigma–Aldrich) and dutasteride (GlaxoSmithKline) at the following concentrations: 0, 0.5, 1, 4, 8, 13, 15, 25, 35, and 50 \(\mu M\), and incubated for 72 h. Each treatment was carried out in triplicate for the given time point.

**MTT assay**

The Methylthiazol Test (MTT), an in vitro toxicity assay (Sigma–Aldrich), was performed to detect cell viability after 72 h of incubation at 37°C. Following the finasteride treatment and incubation described
above, reconstituted MTT solution (15 mg/vial) equal to 10% of the culture medium volume was added to each well of the microtiter plate, and incubated for 4 h at 37°C until the yellow MTT dye was reduced to purple formazan solubilized with acidified isopropanol. The experiment was performed in triplicate. The microtiter plate was then read at an absorbance of 570 nm, and cell viability was examined by dividing the optical density (OD) of the drug-containing wells by the OD of the drug-free control. Appropriate cut off values were determined for the cell lines after the background subtraction at the reference wavelength, 690 nm. The percent cell viability was then calculated as \( \frac{OD_{(treated)} - OD_{(blank)}}{OD_{(control)} - OD_{(blank)}} \times 100\% \).

**Quantitative real-time PCR**

Quantitative real-time PCR (Q-PCR) was performed in FFPE prostate tissue samples \( (n = 3) \) and finasteride-treated, dutasteride-treated, and untreated (control) groups using low-density arrays consisting of genes involved in angiogenesis (each in duplicate) (Applied Biosystems, Foster City, CA, USA). Each Q-PCR reaction constituted of 2\( \times \) TaqMan Universal PCR master mix (10 \( \mu \)l) and cDNA template (15 ng) in a final reaction volume of 20 \( \mu \)l, and was carried out using the ABI Prism 7900HT thermocycler (Applied Biosystems) under standard cycling conditions. Each of the cell lines and tissues was normalized using an endogenous reference, glyceraldehyde-3-phosphate dehydrogenase. For each analysis, a negative control was prepared using all of the reagents except the cDNA template. All reactions were conducted in triplicate. Relative expression of each gene (RQ) was calculated by the comparative cycle threshold method using the formula \( 2^{-\Delta\Delta C_{t}} \), where \( \Delta\Delta C_{t} = \Delta C_{\text{tumor tissue or treated cells}} - \Delta C_{\text{benign tissue or untreated cells}} \) and \( \Delta C_{t} = C_{t} \) (target gene) − \( C_{t} \) (endogenous reference). Taking the standard error and the RQ range into consideration, we established a cut off RQ value or fold change of \( \geq 1.5 \) as ‘upregulation’ of gene expression. For a few genes in the treated cells, gene expression could not be determined. This was most likely due to experimental error, and such genes were designated as ‘undetermined’.

**Nuclear–cytoplasmic extraction from cell lines**

Sub-cellular components (i.e. nucleus and cytoplasm) were separated using a nuclear extraction kit (Active Motif, Tokyo, Japan). Briefly, the cell suspension from the prostate cell lines LNCaP, PC3, and RWPE-1 was centrifuged to separate the supernatant and the pellet. The supernatant was resuspended in 500 \( \mu \)l of 1\( \times \) hypotonic buffer and incubated in ice for 15 min. This was followed by the addition of 25 \( \mu \)l detergent and centrifuged for 30 s at 4°C. This served as the cytoplasmic extract. The pellet represented the nuclear fraction. This nuclear pellet was lysed in 150 \( \mu \)l complete lysis buffer, incubated for 30 min with constant shaking, and centrifuged at 4°C. Nuclear and cytoplasmic extracts were then used for western blotting as described below.

**Western blotting**

Cell lysates extracted from the finasteride- and dutasteride-treated and untreated LNCaP, PC3, and RWPE-1 cell lines were used for detecting the expression of SRD5A1, SRD5A2, VEGF, AR, TGFβ1, and β-actin proteins by western blot analysis. Protein samples were separated using a 12% SDS-PAGE gel and transferred to nitrocellulose membranes. The membranes were pre-treated with 3% BSA and incubated with primary antibodies, namely polyclonal goat anti-human VEGF (dilution 1:800) (Santa Cruz), SRD5A1 (dilution 1:1000) (Santa Cruz), SRD5A2 (dilution 1:2000) (Abnova), AR (dilution 1:2000) (Millipore, Temecula, CA, USA), TGFβ1 (dilution 1:200) (Cell Signaling, Danvers, MA, USA), or β-actin (1:5000) (Santa Cruz) for 1 h. The membranes were then washed in PBS-T (0.1% Tween 20 in PBS) and incubated at room temperature for 30 min with the secondary antibodies specific for each primary antibody. The blots were then visualized with enhanced chemiluminescence with an ECL detection kit (Santa Cruz Biotechnology).

**ELISA**

Expression of VEGF and other proteins in the angiogenesis pathway was measured using the Quantibody Human Angiogenesis Array 2 (RayBiotech Inc., Norcross, GA, USA) in dutasteride-treated and untreated cells (seven treated and three untreated cells). The antibody array is a glass-chip-based multiplexed sandwich ELISA system designed to determine the expression of 30 cytokines simultaneously. Each chip has 16 wells (6 for standards and 10 for samples) with 120 spots per well representing 30 cytokines in quadruplicate. Briefly, samples and standards were added to the wells of the chip array and incubated for 3 h at 4°C. This was followed by three to four washing steps and the addition of primary antibody and HRP-conjugated streptavidin to the wells. The chip was then scanned.
using a Genepix scanner and quantified using Quanti-Body Analyzer software (Ray Biotech Inc). Relative expression levels of the angiogenic proteins in the treated samples were analyzed by comparing the intensities of the signals with respect to the positive and negative controls provided on the chip.

**Statistical analyses**

Statistical analysis was performed using SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). The two-sided $\chi^2$ test or Fisher’s exact test was used to evaluate the correlation between two categorical variables, i.e. for group stages, Gleason scores (GS), and metastasis. The Mann–Whitney $U$ test was used to determine the correlation between pre-operative serum PSA levels and target proteins in the immunohistochemical analysis. Survival analysis was performed according to the Kaplan and Meier method. The Cox proportional hazards regression model was used to analyze the prognostic factors for the clinical and biological characteristics determined at the time of diagnosis. Disease-specific deaths were defined as all deaths presumably caused directly by PC. A $P$ value of $<0.05$ was considered to be significant.

The general linear model was used to evaluate the overall effects of different concentrations of the two inhibitors, finasteride and dutasteride, on percent cell viability. Multiple comparison with Bonferroni adjustment was applied to compare different inhibitor concentrations with the untreated control in the three types of prostate cell lines. A $P$ value of $<0.05$ was considered to be significant.

**Results**

**Expression of target proteins in prostate tissues by immunohistochemistry**

We used immunohistochemistry to examine the expression of the androgen genes SRD5A1, SRD5A2, and AR, as well as one of the major angiogenic proteins, VEGF, in PC and BPH tissues. Whereas both nuclear and cytoplasmic expression in epithelial cells was observed for the isozymes encoded by SRD5A1 (Fig. 1a) and SRD5A2 (Fig. 1b), and for AR protein (Fig. 1c), only cytoplasmic expression was detected for VEGF protein (Fig. 1d) in both PC and BPH tissues. The positive cytoplasmic expression of SRD5A1 was higher in PC compared with BPH (80 vs 65%; $P=0.048$), whereas positive nuclear expression was higher in BPH compared with PC (79 vs 58%; $P=0.012$; Table 2). However, the magnitude of the effects was relatively small. Both the 5α-reductase enzyme types also showed stromal cell expression (Fig. 1a and b).

Endogenous levels of SRD5A enzymes in the nuclear and cytoplasmic components of the prostate

![Figure 1](image-url)
malignant and non-malignant cells were also assessed by western blot. SRD5A1 was highly expressed in both nuclear and cytoplasmic fractions in LNCaP cells, whereas its level was more cytoplasmic than nuclear in PC3 cells and was nuclear only in RWPE-1 cells. The expression of SRD5A2, however, was almost undetectable in LNCaP and RWPE-1 nuclear and cytoplasmic fractions, and was very low in the PC3 nuclear fraction (Fig. 1e).

Correlation between hormonal components and the angiogenic factor showed a statistically significant association between nuclear SRD5A2 and VEGF expression in PC. Tissues that were positive for VEGF and nuclear SRD5A2 were 95 vs 5% respectively in PC (P = 0.012), and 89 vs 11% respectively in BPH (P = 0.001). No such association, however, was observed between SRD5A1 and VEGF in the two tissue types. VEGF expression was significantly associated with nuclear AR expression in PC (69 vs 39%; P = 0.027) and AR expression in BPH (80 vs 20%; P = 0.02). In other words, tumors that showed expression of AR also showed higher levels of VEGF expression, and the expression of these proteins occurred more frequently in PC compared with BPH cases. This is consistent with previous findings (Cheng et al. 2004, Aslan et al. 2005, Boddy et al. 2005).

### Protein expression correlation with clinico-pathological parameters

#### PC group stage

The expression scores of the proteins under investigation, SRD5A1, SRD5A2, AR, and VEGF, in PC (n = 62) were analyzed in two groups, stages I and II (primary stage cancer) and stage III (locally advanced cancer). Nuclear versus cytoplasmic expression of SRD5A1 in tumors was significantly associated with stage III PC (40 vs 80%, P = 0.01, and 60 vs 95%, P = 0.02 respectively) as shown in Table 3. Overall expression of SRD5A2, AR, and VEGF showed no significant difference across the two groups, suggesting that expression of these proteins was maintained throughout early and advanced cancer stages. The target proteins also did not show any significant association with distant metastasis.

### Table 2 Protein expression in the epithelial cells of benign prostatic hyperplasia (BPH) and prostate cancer (PC)

<table>
<thead>
<tr>
<th>Expression of proteins</th>
<th>Nuclear</th>
<th></th>
<th></th>
<th></th>
<th>Cytoplasmic</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BPH (%)</td>
<td>PC (%)</td>
<td>P value</td>
<td>BPH (%)</td>
<td>PC (%)</td>
<td>P value</td>
<td>BPH (%)</td>
<td>PC (%)</td>
</tr>
<tr>
<td>SRD5A1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>N=48</td>
<td>N=103</td>
<td>0.01*</td>
<td>N=48</td>
<td>N=103</td>
<td>0.048*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>21</td>
<td>42</td>
<td></td>
<td>35</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRD5A2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>N=46</td>
<td>N=106</td>
<td>0.12</td>
<td>N=46</td>
<td>N=106</td>
<td>0.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>61</td>
<td>74</td>
<td></td>
<td>35</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>N=46</td>
<td>N=106</td>
<td>0.12</td>
<td>N=46</td>
<td>N=106</td>
<td>0.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>39</td>
<td>26</td>
<td></td>
<td>65</td>
<td>71</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>N=52</td>
<td>N=108</td>
<td>0.09</td>
<td>N=52</td>
<td>N=108</td>
<td>0.01*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>71</td>
<td>57</td>
<td></td>
<td>83</td>
<td>63</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>43</td>
<td></td>
<td>17</td>
<td>37</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P value < 0.05 is significant.

### Table 3 Clinical characteristics compared with nuclear and cytoplasmic protein expression

<table>
<thead>
<tr>
<th>Clinical characteristic</th>
<th>Nu</th>
<th>Cyt</th>
<th>Nu</th>
<th>Cyt</th>
<th>Nu</th>
<th>Cyt</th>
<th>Nu</th>
<th>Cyt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group stages (I and II/III)</td>
<td>40/80</td>
<td>60/95</td>
<td>35/27</td>
<td>65/91</td>
<td>–</td>
<td>84/77</td>
<td>42/45</td>
<td>21/45</td>
</tr>
<tr>
<td>P value</td>
<td>0.01*</td>
<td>0.02*</td>
<td>0.59</td>
<td>0.06</td>
<td>0.70</td>
<td>0.83</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>Metastasis (M0/M1)</td>
<td>62/80</td>
<td>79/87</td>
<td>29/20</td>
<td>77/73</td>
<td>79/62</td>
<td>44/25</td>
<td>37/44</td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>0.20</td>
<td>0.71</td>
<td>0.74</td>
<td>0.76</td>
<td>0.31</td>
<td>0.18</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td>Gleason score (4–6/7/8–10)</td>
<td>42/73/83</td>
<td>63/83/97</td>
<td>26/37/21</td>
<td>63/100/71</td>
<td>83/87/60</td>
<td>33/37/44</td>
<td>33/44/39</td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>0.02*</td>
<td>0.05</td>
<td>0.51</td>
<td>0.03*</td>
<td>0.08</td>
<td>0.78</td>
<td>0.82</td>
<td></td>
</tr>
<tr>
<td>Pre-operative serum PSA level</td>
<td>23</td>
<td>20</td>
<td>10</td>
<td>20</td>
<td>89</td>
<td>23</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Median (µg/l)</td>
<td>23</td>
<td>20</td>
<td>10</td>
<td>20</td>
<td>89</td>
<td>23</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>0.01*</td>
<td>0.002*</td>
<td>0.25</td>
<td>0.32</td>
<td>0.25</td>
<td>0.87</td>
<td>0.12</td>
<td></td>
</tr>
</tbody>
</table>

*P value < 0.05 is significant (Leung et al. 1989).
Protein expression, when analyzed in three groups of GS, showed increased SRD5A1 nuclear expression in GS 8–10 as compared with GS 7 and GS 4–6 of PC, which was statistically significant (83 vs 73 vs 42%, \( P = 0.02 \)). SRD5A2 cytoplasmic expression was, however, more frequent in the GS 7 group than in the GS 4–6 and GS 8–10 groups (71 vs 100 vs 63%, \( P = 0.03; \) Table 3).

Pre-operative serum PSA levels
SRD5A1 nuclear and cytoplasmic expression (median 23 and 20 \( \mu \)g/l respectively, range 2.0–4864 \( \mu \)g/l) showed significant association with elevated pre-operative serum PSA levels (Table 3). The other three proteins, SRD5A2, VEGF, and AR, did not correlate with PSA levels. Survival analysis did not indicate any statistical significance among these biomarkers (data not shown).

Expression of angiogenesis pathway genes in PC tissues by Q-PCR
We also carried out Q-PCR using low-density arrays to examine the expression of \( SRD5A1, \) \( SRD5A2, \) \( AR, \) and a panel of genes in the angiogenesis pathway in prostate tumor FFPE samples \( (n = 3) \) that had already been analyzed immunohistochemically. The samples were of the same grade (GS=7) but different stages (T2, T3, and T4, one of each). The fold changes of the genes in PC tissues are shown in Table 4. Gene expression was upregulated in the metastatic stage (T4) compared with the other two stages (T2 and T3) in the PC tissues, confirming their expression.

Cell viability assay in finasteride and dutasteride treatment groups
We next performed an \textit{in vitro} analysis to study the molecular effects of SRD5A inhibitors in the angiogenesis pathway. For this, we first examined whether or not inhibition of 5\( \alpha \)-reductase activities could attenuate the proliferation of prostate malignant and non-malignant cells. We treated the malignant LNCaP and PC3, and non-malignant RWPE-1, cells with finasteride and dutasteride for 72 h. Since a time- and dose–response experiment for dutasteride had already been conducted by \textit{Schmidt et al.} (2004), demonstrating that viability and morphology of the androgen-dependent LNCaP cells was affected after 48 h of treatment, we chose a longer time period of 72 h treatment for our experiments.

The MTT assay demonstrated a significant difference in cell viability between finasteride-treated cells and untreated controls in the three cell types. The LNCaP cells showed a reduction in cell viability as the treatment concentration increased. The cell viability was reduced to 65% at 4 \( \mu \)M, and was reduced to 8% at 13 \( \mu \)M. This was statistically significant when compared with untreated controls \( (P < 0.0005; \) Fig. 2a). In PC3-treated cells, cell numbers were also reduced noticeably at concentrations between 4 and 50 \( \mu \)M. Cell viability at 4 \( \mu \)M was 57%, and dropped to 36% at 25 \( \mu \)M. In RWPE-1 cells, growth was inhibited significantly at concentrations between 35 and 50 \( \mu \)M compared with untreated control cells \( (P < 0.0005). \) Though not statistically significant, cell viability reductions of up to 81% were observed at 4–50 \( \mu \)M finasteride, with a 65% reduction at the 25 \( \mu \)M concentration.

In the dutasteride-treated LNCaP group, a reduction in cell number was evident starting at the first dosage of 0.5 \( \mu \)M dutasteride; at this dose, cell viability was reduced to 88%. Then, at 1 \( \mu \)M, cell viability was further reduced to 79%, and at 25 \( \mu \)M, viability was reduced to 24%, a statistically significant reduction \( (P < 0.0005) \) compared with the untreated controls. Cell numbers were also reduced in PC3 cells; at 0.5 \( \mu \)M dutasteride, cell viability was reduced to 90%. As with LNCaP cells, higher doses of dutasteride resulted in more significant reductions in viability: at
viability was reduced to 58%, and at 25 μM, viability was reduced to 2%. In RWPE-1 cells, dutasteride was also effective in inhibiting cell growth at doses ranging from 0.5 to 50 μM (P < 0.0005), with a reduction to 55% cell viability at 0.5 μM and 11% at 25 μM concentrations.

We also compared the overall effects of finasteride and dutasteride treatment at different concentrations on cell viability in the three cell types (Fig. 2a and b). No statistically significant differences were observed between the two inhibitors in LNCaP-treated cells (P = 0.520), but in PC3 and RWPE-1 cells, dutasteride was significantly more effective in inhibiting cell growth than finasteride (P < 0.0005).

Expression of angiogenesis pathway genes in treated PC cells

Using Q-PCR, we next examined the effect of finasteride and dutasteride at 1 and 25 μM on the expression level of different genes in the angiogenesis pathway. By examining RNA isolated from treated and untreated cells, expression of our gene set was compared using low-density arrays. The two doses were chosen, based on the results of the viability assay, to show the molecular effects of treatment at a low concentration of 1 μM and at a relatively high concentration of 25 μM.

In LNCaP cells treated with 1 and 25 μM finasteride and dutasteride, the angiogenic genes endothelin (EDN1), VEGFR1, TGFA, and TGFB1 showed increased expression compared with the untreated controls (Table 5). The expression of AR was also increased at the 1 μM dose, but was reduced (<1.5-fold) at 25 μM, for both inhibitors. However, the expression of the genes was not statistically significant.

In finasteride-treated PC3 cells, the expression of the genes did not follow that of the androgen-responsive LNCaP cells. At the 1 μM concentration, most of the genes were downregulated, with the exception of interleukin (IL)-7R. However, at a concentration of 25 μM finasteride, at least 7 of the 21 genes tested were upregulated (Table 5). In dutasteride-treated PC3 cells, downregulation of all of the genes was observed at both 1 and 25 μM concentrations, with the exception of TGFB1, although the expression was not statistically significant (P = 0.33) probably due to smaller number of the samples.

In RWPE-1 cells treated with 1 μM finasteride, the expression of hypoxia-inducible factor 1 α (HIF-1α), IL-6, IL-15, serine peptidase inhibitor (SERPINB5), TGFA, TGFB, TGFB2, transforming growth factor β receptor 1 (TGFBR1), TGFBR3, VEGFA, and SRD5A1 genes showed a fold change ≤1.4-fold. Furthermore, the expression of these genes was lower in cells treated with 25 μM finasteride doses compared with 1 μM doses. In the dutasteride-treated cells, the expression of all of the genes was lower than that in the untreated cells for both 1 and 25 μM concentrations.

Expression of angiogenesis pathway proteins in treated PC cells

Using ELISA with a human angiogenesis antibody array, we also examined the expression of proteins involved in the angiogenesis pathway. Though expression of VEGF was reduced at all concentrations (0.5–50 μM) of finasteride in LNCaP cells, the
Table 5 Expression of genes in the angiogenesis and androgen-signaling pathways in LNCaP- and PC3-treated cells

<table>
<thead>
<tr>
<th>Genes</th>
<th>LNCaP cells</th>
<th>PC3 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Finasteride</td>
<td>Dutasteride</td>
</tr>
<tr>
<td></td>
<td>1 μM</td>
<td>25 μM</td>
</tr>
<tr>
<td>CDH1</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>EDN1</td>
<td>2.7*</td>
<td>1.9*</td>
</tr>
<tr>
<td>VEGFR1</td>
<td>1.7*</td>
<td>1.9*</td>
</tr>
<tr>
<td>HIF-1A</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>IL-6</td>
<td>-2.9</td>
<td>-1.2</td>
</tr>
<tr>
<td>IL-7R</td>
<td>-1.2</td>
<td>-1.1</td>
</tr>
<tr>
<td>IL-15</td>
<td>-5</td>
<td>-1.8</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>-10</td>
<td>1.0</td>
</tr>
<tr>
<td>SERPINB5</td>
<td>-14.3</td>
<td>-3.1</td>
</tr>
<tr>
<td>SERPINB8</td>
<td>1.1</td>
<td>-1.8</td>
</tr>
<tr>
<td>TGFA</td>
<td>1.5*</td>
<td>1.5*</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>2.2*</td>
<td>2.1*</td>
</tr>
<tr>
<td>TGFβ2</td>
<td>-1.0</td>
<td>0.7</td>
</tr>
<tr>
<td>TGFβ3</td>
<td>-1.1</td>
<td>0.4</td>
</tr>
<tr>
<td>TGFβR1</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>TGFβR2</td>
<td>1.2</td>
<td>-1.1</td>
</tr>
<tr>
<td>TGFβR3</td>
<td>1.1</td>
<td>1.5*</td>
</tr>
<tr>
<td>VEGFA</td>
<td>1.4</td>
<td>-1.1</td>
</tr>
<tr>
<td>AR</td>
<td>1.5*</td>
<td>1.2</td>
</tr>
<tr>
<td>SRD5A1</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>SRD5A2</td>
<td>UD</td>
<td>UD</td>
</tr>
</tbody>
</table>

UD, undetermined; * denotes upregulation.

difference between control and treatment groups was not statistically significant (P=0.085; Fig. 3a). In PC3 cells, however, VEGF levels increased at higher finasteride concentrations (35–50 μM) compared with untreated controls, and the difference between the treated and control group was statistically significant (P<0.0005; Fig. 3a). In RWPE-1 cells, VEGF levels were considerably increased with finasteride doses ranging from 1 to 50 μM, but there was no statistical difference between different dosages compared with the controls (P=0.094).

In the dutasteride treatment group, the expression level of VEGF in the LNCaP and PC3 cells was reduced compared with the untreated controls. The difference was statistically significant in PC3 cells (P<0.0005) and nearly significant in LNCaP cells (P=0.059; Fig. 3a). Western blot analysis indicated a similar pattern of expression of VEGF protein in finasteride- and dutasteride-treated LNCaP and PC3 cells (Fig. 3b). In RWPE-1 cells, however, no statistical difference was observed between VEGF at 25–50 μM and the untreated controls.

The Quantibody array analysis detected expression of several proteins essential for hormone-mediated signaling in treated groups of all three cell types (LNCaP, PC3, and RWPE-1). These proteins included a neuropeptide, agouti-related protein; a vasculo-genesis protein, angiopoietin-2 (ANG-2); a cytokine, IL1-A; growth factors basic fibroblastic growth factor and insulin-like growth factor 1; and the tissue inhibitor of metalloproteinase 2 (TIMP-2). The expression of these proteins, however, was lower than that in the untreated controls. Expression of the chemokines epithelial cell-derived neutrophil-activating peptide (ENA-78) and growth-related chemokine (GRO) and the collagenase inhibitor TIMP-1 was observed only in the PC3 cells. Expression of the C-C motif chemokine monocyte chemotactic protein-1 (MCP-1) and the growth factors heparin-binding EGF-like growth factor (HB-EGF) and platelet-derived growth factor β polypeptide (PDGF-BB), on the other hand, was observed only in the LNCaP cells, and the expression of these proteins was lower in the treated cells compared with the untreated controls. However, expression of the transforming growth factor TGFβ1 was higher in dutasteride-treated LNCaP cells than in controls (Fig. 3c). This was also confirmed by western blot (Fig. 3b). Similarly, in PC3 cells, TGFβ1 and IL-6 expression was also increased in the treated groups versus the controls, but IL-8 expression was
comparable to that of the controls. None of the other proteins (Activin A, Angiogenin, angiopoietin-like 4 (ANGPTL4), hepatocyte growth factor (HGF), IL-2, IL-17, chemokine (C-X-C motif) ligand 10 (IP-10), Leptin, leukemia inhibitory factor, PIGF, CD8-positive T lymphocytes (RANTES), tumor necrosis growth factor α (TNFA), TNFB, thyroid peroxidase, and interferon γ) could be detected in the dutasteride-treated groups in any of the three cell lines investigated.

Discussion

It has previously been demonstrated that the two important isoforms of SRD5A, encoded by the SRD5A1 and SRD5A2 genes, which convert testosterone into the more bioactive DHT in the prostate, are required in greater amounts to suppress the activity of DHT in malignant versus non-malignant prostatic epithelial cells (Ellis & Isaacs 1985, Lamb et al. 1992). However, which of the two isoforms plays the major role in PC is still debatable. Because there are discrepancies regarding the presence and significance of SRD5A1 in the prostate due to variability in its expression (Habib et al. 2003, Thomas et al. 2003, 2005, Nakamura et al. 2005, Titus et al. 2005, Wako et al. 2008), we decided to investigate the sub-cellular expression level of the isozymes in BPH versus PC in human tissues of different pathological grade and stage.

In the present study, we show distinct nuclear and cytoplasmic expression of SRD5A1 in human PC tissues and a significant correlation between nuclear SRD5A1 expression and higher stage, GS, and pre-operative serum PSA levels, suggesting that this is the predominant isozyme during PC progression. Although increased expression of SRD5A1 in prostate tumor tissue has been reported previously (Thomas et al. 2005, Wako et al. 2008), the importance of its sub-cellular localization in PC has not been clearly demonstrated. The activity of DHT in the prostate is based on nuclear and cytoplasmic localization of SRD5A. Considering an earlier finding that approximately half of the 5α-reductase enzyme responsible for the transformation of testosterone to DHT is located in the nuclei of the prostate cell (Gustafsson & Pousette 1974, Enderle-Schmitt et al. 1986), it was necessary to investigate the nuclear presence of the enzyme in PC cells. Although SRD5A was shown to be localized in the nuclear membrane (an integral membrane protein; Enderle-Schmitt et al. 1986), we also observed localization of the enzyme within the nucleus. This could be due to the fact that the prostatic enzyme is dependent on both polar and non-polar portions of the surrounding phospholipids, and that modifications of the phospholipids surrounding this enzyme could have
resulted in alterations in enzymatic activity, resulting in localization to the nucleus. Since DHT formed in the cytoplasm by cytoplasmic SRD5A could be subject to several other reductive and oxidative metabolic pathways, the less-varied nuclear DHT formed by nuclear SRD5A is much more readily available to be transported to the intranuclear target site (Gustafsson & Pousette 1974). Therefore, our finding of an association between nuclear SRD5A1 and advanced PC suggests that this isozyme may activate large amounts of androgens during its transport from the cytoplasm to the nucleus, affecting transcriptional events in the cell to bring about highly aggressive tumors. We also confirmed expression of SRD5A1 in human prostate malignant and non-malignant cell lines. This was in accordance with a previous report (Xu et al. 2006), although we examined sub-cellular protein expression, whereas they examined mRNA levels of SRD5A1 in these cells.

Conversely, we were unable to establish a strong association between SRD5A2 expression and GS or stage in PC tissues. This likely reflected the biological function of SRD5A2 in regulating DHT level during tumorigenesis, so that continued expression of this isoform once cancer was initiated did not have to correlate with histological appearances. SRD5A2 expression in the PC tissues did not correlate with that in cell lines, either, with almost undetectable levels of SRD5A2 in the cell lines. The presence of stromal cells in the tissues, but not in the cell lines, may explain this discrepancy, as also suggested by Xu et al. (2006).

It is now widely accepted that PC growth and progression are characterized not only by high DHT levels in the prostate but also by a high degree of vascularization (Folkman 1995). We were interested in studying both hormonal and vascular components to determine the relationship between steroids and PC cell angiogenic responses. We chose VEGF as a marker of angiogenesis, as it is considered the most important regulator of angiogenesis (Ferrara et al. 1992). We verified the pattern of distribution of VEGF in PC in vivo, and then correlated the VEGF expression level with levels of the SRD5As and AR. A statistically significant association between VEGF, SRD5A2, and AR was evident in the PC tissues, but not between VEGF and SRD5A1, suggesting that the type II isoform may regulate the expression of VEGF mediated by AR during tumorigenesis.

Q-PCR results examining different cancer stages, but of the same GS, indicated that expression of a panel of genes in the angiogenesis pathway increased according to disease aggressiveness. Consequently, these data were used to further understand the role of SRD5A in PC cell angiogenic responses in our experimental in vitro model by using SRD5A inhibitors.

When the effects of the inhibitors on cell viability were compared in the three treated cell lines, dutasteride was more effective at overall inhibition of cell growth in PC3 and RWPE-1 cells compared with LNCaP cells, although LNCaP cells were inhibited at a lower concentration (0.5 μM) of dutasteride. Schmidt et al. (2004) demonstrated a reduction in LNCaP cell viability and cell numbers only at 1 μM of dutasteride after 48 h of treatment, in agreement with our observation at 72 h of treatment. Dutasteride was, however, shown to be more effective at inhibiting LNCaP cell growth compared with finasteride in another study (Lazier et al. 2004). The discrepancy in the results could be due to the passage number, as our cells were at an early passage of <20.

Our low-density array data revealed continuously increased expression of TGFB1 in both finasteride- and dutasteride-treated LNCaP and PC3 cells, although the expression was not statistically significant. Expression was, however, lower in PC3 than in LNCaP cells, and expression was confirmed by western blot in LNCaP cells (Fig. 3b). In addition, Quantibody array analysis showed greater amounts of TGFB1 with higher concentrations of dutasteride treatment in LNCaP and PC3 cells compared with untreated controls (Fig. 3c). TGFB1 is known to be frequently upregulated in tumor cells. Increased expression of this gene has been suggested to affect the tumor microenvironment, resulting in tumor growth promotion, invasion, and angiogenesis (Ueki et al. 1992, Hanahan & Weinberg 2000, Derynck et al. 2001). On the other hand, stimulation of TGFB1 is also known to suppress tumor growth (Moses et al. 1990). Thus, besides participating in decreased cell viability in our experimental model, increased expression of TGFB1 at higher concentrations of the inhibitors could also be suggestive of tumor resistance to the inhibitors.

We also observed increased expression of other genes, including EDN1, TGFα, and the VEGF receptor FLT1, in the treated LNCaP cells, and VEGF, HIF-1, and IL-6 in the treated PC3 cells. It has been reported that growth factors such as VEGF and TGF and their receptors induce other signaling pathways (PI3K, Smad, etc.) that result in endothelial cell proliferation, increased vascular permeability, and cell migration (Karkkainen & Petrova 2000). The increased expression of VEGF in our study could be either due to increased TGFB1 expression inducing VEGF to act directly on endothelial cells and stimulate cell proliferation and migration (Pertovaara et al. 1994),
or due to increased expression of AR, as AR has been shown to regulate VEGF in PC (Zhu & Kyprianou 2008). Another possible reason could be the extended or longer duration of treatment, since increased expression levels of the above genes were not reported by Schmidt et al. (2004). The exposure of the tumor cells to the inhibitors for 72 h might have changed the tumor microenvironment, resulting in evasive resistance to these inhibitors by the tumors, which has also been reported recently for VEGF inhibitors (Loges et al. 2009, Paez-Ribes et al. 2009). Our study also demonstrated upregulation of AR gene expression in LNCaP cells, which might have been the result of the sudden depletion in the level of DHT; this depletion could have stimulated AR to bind with other available ligands.

Our experimental findings in the LNCaP cells for the two inhibitors may not be therapeutically promising. However, differential expression of the angiogenesis pathway genes at the same concentration of the two inhibitors may suggest that the effects of the two SRD5A isoforms on angiogenesis genes are similar. Of course, dutasteride was able to suppress tumor growth more efficiently than finasteride, as described in our viability assay, possibly also due to SRD5A1 inhibition.

Since the aim of the present study was to demonstrate the biological role of the 5α-reductase isoforms and identify their relationship with the genes and proteins in the angiogenesis pathway, we therefore conclude that we have observed the predominance of SRD5A1 in the more advanced tumor tissues and cell lines, and its inhibition along with SRD5A2 was more effective in reducing cell numbers in the prostate tumor cells than SRD5A2 inhibitor alone. However, the expression level of our target angiogenic genes and proteins in the presence of SRD5A isoform, SRD5A1 (inhibiting SRD5A2), and in the absence of SRD5A1 and SRD5A2 did not show any significant difference, and few of the target genes were still upregulated for which duration of treatment and tumor resistance to the inhibitors could not be ruled out.

Since endogenous levels of SRD5A2 expression were undetectable in the PC cells, and since treatment with the inhibitor of the type II isozyme showed molecular effects both before and after treatment, we think a better model than the cell lines should be used for further analysis. Therefore, future studies may consider using a different experimental model, such as a transgenic mouse model, to study the efficacy of these inhibitors and also to understand the relationship between angiogenesis and androgen signaling pathways in tumor progression and metastasis.

### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

### Funding

This work was supported by an A*Star Singapore Cancer Syndicate Grant (MN05-R) and a Cancer Science Institute Grant (R-713-002-015-271) to M Salto-Tellez.

### References


Lazier CB, Thomas LN, Douglas RC, Vessey JP & Rittmaster RS 2004 Dutasteride, the dual 5alpha-reductase inhibitor, inhibits androgen action and promotes cell death in the LNCaP prostate cancer cell line. Prostate 58 130–144.


