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5α-Reductase Type 3 Expression in Human Benign and Malignant Tissues: A Comparative Analysis During Prostate Cancer Progression

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Abstract

BACKGROUND—A third isozyme of human 5α-steroid reductase, 5α-reductase-3, was identified in prostate tissue at the mRNA level. However, the levels of 5α-reductase-3 protein expression and its cellular localization in human tissues remain unknown.

METHODS—A specific monoclonal antibody was developed, validated, and used to characterize for the first time the expression of 5α-reductase-3 protein in 18 benign and 26 malignant human tissue types using immunostaining analyses.

RESULTS AND CONCLUSIONS—In benign tissues, 5α-reductase-3 immunostaining was high in conventional androgen-regulated human tissues, such as skeletal muscle and prostate. However, high levels of expression also were observed in non-conventional androgen-regulated tissues, which suggest either multiples target tissues for androgens or different functions of 5α-reductase-3 among human tissues. In malignant tissues, 5α-reductase-3 immunostaining was ubiquitous but particularly over-expressed in some cancers compared to their benign counterparts, which suggests a potential role for 5α-reductase-3 as a biomarker of malignancy. In benign prostate, 5α-reductase-3 immunostaining was localized to basal epithelial cells, with no immunostaining observed in secretory/luminal epithelial cells. In high-grade prostatic intraepithelial neoplasia (HGPIN), 5α-reductase-3 immunostaining was localized in both basal epithelial cells and neoplastic epithelial cells characteristic of HGPIN. In androgen-stimulated and

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castration-recurrent prostate cancer (CaP), 5α-reductase-3 immunostaining was present in most epithelial cells and at similar levels, and at levels higher than observed in benign prostate. Analyses of expression and functionality of 5α-reductase-3 in human tissues may prove useful for development of treatment for benign prostatic enlargement and prevention and treatment of CaP.

Keywords

5α-reductases; androgen metabolism; dihydrotestosterone; benign prostatic hyperplasia; castration-recurrent prostate cancer

INTRODUCTION

Androgen target cells use testosterone (T) as a prohormone, where T is converted by intracrine pathways into dihydrotestosterone (DHT), the most potent androgen receptor (AR) ligand. The enzyme steroid 5α-reductase (EC1.3.99.5) [1] localizes DHT biosynthesis to androgen responsive tissues. 5α-reductase isozymes 1 and 2 are well characterized [2] and DHT levels in target tissues are reduced using 5α-reductase inhibitors. However, male pattern baldness, that affects an estimated 70% of men by age 80 [3] responds poorly to 5α-reductase-2 inhibition (finasteride, Propecia™) [4]. Lower urinary tract symptoms from benign prostate enlargement affect 50% of men age 50 or older [5], and 20% of men treated with the 5α-reductase-2 inhibitor, finasteride (Proscar™), or the bispecific inhibitor of 5α-reductase-1 and 2, dutasteride (Avodart™), require operative treatment within 4 years [6]. CaP is the most common non-skin cancer in American men; approximately 217 730 Americans will be diagnosed with CaP and approximately 32 050 men will die from CaP in 2010 [7]. Finasteride treatment of men at increased risk of CaP decreased the diagnosis of CaP by 25%, however, the CaP that developed often was more poorly differentiated than observed in controls [8]. Dutasteride decreased the incidence of diagnosis of CaP and did not affect differentiation [9], but may cause delay of diagnosis that is concerning especially for aggressive CaP [10].

Men who fail curative therapy for clinically localized CaP or are diagnosed with advanced disease usually receive androgen deprivation therapy (ADT) that causes regression of androgen-stimulated CaP through programmed cell death [11]. However, ADT is palliative since CaP almost always recurs and causes death. A molecular role for AR in the transition from androgen-stimulated CaP to castration-recurrent CaP is supported by continuous expression of AR [12-14] and androgen-regulated genes [15]. Many alternative mechanisms allow AR-mediated transactivation of gene expression despite castrate levels of circulating testicular androgens (reviewed by Refs. 16-23). However, we and others have demonstrated that castration-recurrent CaP maintains tissue levels of DHT sufficient for activation of even wild-type AR [24-26]. Limited clinical experience has not demonstrated efficacy for finasteride added to ADT for advanced CaP [27] or for dutasteride or LY320236, another bi-specific 5α-reductase inhibitor [28], for secondary treatment of castration-recurrent CaP. Consequently, castration-recurrent CaP must be capable of biosynthesis of intraprostatic T from circulating adrenal androgens [29] or cholesterol [30] and metabolism of T to DHT at levels sufficient for AR activation by incompletely characterized mechanisms.

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Laboratory observations and clinical experience led to speculation about the existence of an additional 5α-reductase isozyme. A candidate sequence consistent with a 5α-reductase isozyme that mapped to chromosome 4q12 was identified by mining the Human Genome Project database [31]. Uemura et al. [32] confirmed the existence of a novel 5α-steroid reductase (SRD5A3, type-3) in human tissues but only at the mRNA level. Our study is the first to analyze the expression of 5α-reductase-3 protein in human benign and malignant tissues. In this analysis, special emphasis was given to the expression of 5α-reductase-3 during prostate carcinogenesis and progression.

MATERIALS AND METHODS

Cell Culture

PWR-1E, a benign human prostate epithelial cell line, was purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in Kerationocyte Serum Free media (Invitrogen, Carlsbad, CA) supplemented with 5 ng/ml recombinant EGF and 0.05 mg/ml bovine pituitary extract (Gibco, Carlsbad, CA). LNCaP and C4-2 cells were maintained in RPMI-1640 supplemented with 2 mM glutamine and 10% fetal bovine serum (FBS, Mediatech, Inc, Herndon, VA). LAPC-4 was maintained in RPMI-1640 supplemented with 10 nM R1881 (Perkin-Elmer, Boston, MA). CWR-R1 was maintained in Richter's Improved MEM (Mediatech) supplemented with 20 mM HEPES, 5 mg/ml insulin, 5 mg/ml transferin, 5 ng/ml sodium selenite (ITS, Roche, Indianapolis, IN), 0.1 mg/ml epidermal growth factor (Invitrogen), 1 mg/ml licoleic acid, 10 mM nicotinamide (Sigma–Aldrich, St. Louis, MO), and 2% FBS. CHO-K1, a subclone from the parental CHO cell line, was purchased from ATCC and maintained in Nutrient Mixture F12K Kaighn's modification media (Invitrogen) supplemented with 2 mM L-glutamine and 10% FBS.

Anti-5α-reductase-3 Polyclonaland Monoclonal Antibodies

A commercially available polyclonal antibody produced by Sigma (SRD5A3, Sigma–Aldrich) and a total of four rabbit polyclonal antibodies produced commercially (Open Biosystems, Huntsville, AL) against amino acids 1–16, 3–18, 46–62, and 48–65 of the N-terminus of the 5α-reductase-3 protein were evaluated using Western blot and immunohistochemistry and the polyclonal antibody targeting the first epitope, $M^{1}APWAEAEHSALNPLR^{16}$ performed best. Two hybridomas were produced in our laboratory against the same epitopes within the N-terminus and the RPCI-5αR3 monoclonal antibody performed best. RPCI-5αR3 antibody resulted from fusion of murine myeloma (P3X63 Ag8U.1, ATCC CRL 1597) with spleen cells of mouse hosts immunized with synthetic peptide ($M^{1}APWAEAEHSALNPLR^{16}$) using a standard procedure [33]. Each peptide was coupled to keyhole limpet hemocyanin using the 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride method (Pierce). Hybridomas were cloned twice using the limiting dilution method [34]. Stable hybridomas that produced monoclonal antibodies were expanded and cryopreserved. Hybridoma cells were injected (1×10^{7}) into the peritoneal cavity of female SCID mouse hosts to produce ascites fluid. All animal studies were performed in compliance with US Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and approved by Roswell Park Cancer Institute Animal Care and Use Committee. Ascites fluid was harvested 3–4 weeks

after hybridoma cells were injected when it contained 1.2–4 mg/ml of monoclonal antibody. Monoclonal antibodies were purified from ascites on Affi-gel protein A agarose (Bio-Rad) following manufacturers instructions. RPCI-5αR3 antibody was of the IgG-1 subclass, as determined using an ImmunoPure Monoclonal Antibody Isotyping Kit (Pierce). The immunoreactivity of the RPCI-5αR3 antibody with prostate cells was confirmed; crude extracts (50 μg) prepared from LNCaP, C4-2, LAPC-4, and CWR-R1 cells were reacted with synthetic peptide (20 ng) at 4°C for 2 hr. The proteins were separated using SDS-Page and electrophoretically transferred onto a polyvinylidene fluoride membrane. Membranes were developed using the RPCI-5αR3 monoclonal or polyclonal antibodies, horseradish peroxidase (HRP), and electrochemoluminescence. The specificity of each antibody also was tested using an enzyme-linked immunosorbent assay (ELISA) and Western blot. The detection antigen was peptide conjugated with bovine serum albumin (BSA, EMD Chemicals, Gibbstown, NJ).

Reverse Transcription and Quantitative Real-Time PCR

Total RNA from CWR-R1 cells was isolated using RNeasy Mini kits (Qiagen). Total RNA (400 ng) was reverse-transcribed into cDNA using random primers (Invitrogen). Primers and probes for 5α-reductase-3 were purchased from Applied Biosystems. PCR reactions were performed using the 7300 Real-Time PCR system (Applied Biosystems) with a total reaction mixture volume of 25 μL containing 8.0 ng cDNA, 1× TaqMan Universal PCR Master Mix (Applied Biosystems) and 1× primers and probe mix. The PCR conditions for all reactions were: 95°C for 10 min, 40 amplification cycles at 95°C for 15 sec and 60°C for 1 min. The relative mRNA levels of 5α-reductase-3 in LNCaP, C4-2, LAPC-4, and CWR-R1 were expressed relative to PWR-1E.

Western Blot

Total cell extracts (50 μg of total protein) from PWR-1E, LNCaP, C4-2, LAPC-4, CWR-R1, and CHO-K1, were separated electrophoretically using SDS-poly-acrylamide gel electrophoresis (10% w/v; Bio-Rad Laboratories, Hercules, CA) under reducing conditions and the separated proteins transferred to nitrocellulose membranes using standard procedures [35,36]. Western analyses utilized as primary antibody the RPCI-5αR3 (1 μg/ml) or the SRD5A3 (1 and 5 μg/ml) antibody. HRP-conjugated anti-mouse or anti-rabbit IgG (Dako) was used as secondary antibody, respectively. Antibody localization was visualized using enhanced chemi-luminescence (Pierce Biotechnology). CHO-K1 cell line was used as negative control for expression of 5α-reductase-3 [37]. Equal loading of total cell extracts was verified visually by immunohistochemical staining of membranes with anti-β-actin (Santa Cruz Biotechnology).

5α-Reductase-3 Immunostaining

CWR-R1 cells were fixed in situ for 30 min at room temperature using 4% w/v paraformaldehyde. Endogenous peroxidase activity was inhibited with 3.0% v/v H_2O_2 in methanol. Antibody specificity was evaluated by pre-incubating the monoclonal anti-body with the inhibitor peptide for 2 hr before immunostaining. Tissue microarrays from benign and malignant human tissues were obtained from the Roswell Park Cancer Institute Department of Pathology. In addition, prostate tissue sections were cut from formalin-fixed,

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paraffin-embedded clinical specimens of androgen-stimulated benign prostate (BP) (n = 8), high-grade prostatic intraepithelial neoplasia (HGPIN), (n = 8) and androgen-stimulated CaP (n = 8) obtained from the Department of Pathology archives or from a tissue microarray constructed from formalin-fixed, paraffin-embedded clinical specimens of androgen-stimulated BP (n = 18), androgen-stimulated CaP (n = 21) and castration-recurrent (n = 19), CaP which has been studied previously [14,22,38-45]. All tissues and the images obtained from these tissues for analysis were reviewed by one pathologist (Borislav Alexiev). In tissue sections, antigens were retrieved using microwave irradiation and citrate buffer pH 6.0 for 15 min. Endogenous peroxidase activity was inhibited using 3% v/v H_2O_2 in methanol. Sections and CWR-R1 cells were incubated overnight with the RPCI-5αR3 antibody diluted 1:100 (optimum by checkerboard analysis of 1:50–1:1000 dilutions) in 100 mM Tris-HCl buffer (pH 7.8) that contained 8.4 mM sodium phosphate, 3.5 mM potassium phosphate, 120 mM NaCl, and 1% w/v BSA. After washing 3 times for 10 min in Tris-HCl buffer (pH 7.8), sections were incubated with HRP-conjugated anti-mouse IgG (1/100, Dako, Carpinteria, CA) secondary antibody for 2 hr at room temperature. Peroxidase activity was developed using 100 mM Tris-HCl buffer containing 3,3-diaminobenzidine tetrahydrochloride (1 μg/ml, Sigma-Aldrich) and H_2O_2 (1 μl/ml, VWR International, West Chester, PA). Colocalization analyses of 5α-reductase-3/p63 and 5α-reductase-3/alpha-methylacyl-coenzyme-A racemase (AMACR) were performed using the EnVision™G/2 Double Stain System according to the manufacturer's instructions (Dako) [46]. 5α-Reductase-3 was detected using an HRP-conjugated secondary antibody and 3,3-diaminobenzidine tetrahydrochloride as substrate (brown precipitate) and p63 and AMACR protein expression were visualized using an alkaline phosphatase-conjugated secondary antibody and Permanent Red substrate (red precipitate). Sections were counterstained using hematoxylin. Immunohistochemistry in absence of primary antibody provided negative controls.

Digital Image Collection and Analysis

For cell lines and tissue sections (including tissue microarrays), images were collected using an Hamamatsu Color Chilled 3CCD camera (Hamamatsu, Bridgewater, NJ) mounted on an Axioskop microscope (Carl Zeiss, Thornwood, NY). For the prostate tissue microarray, five images were collected from each 2.0 mm core at total magnification 400×. The images were arranged in random order in a digital album using Adobe Photoshop (Adobe Systems, San Jose, CA) to facilitate visual scoring. Images were visually scored by three experienced observers (M. A. Titus, O. H. Ford and J. L. Mohler) who were blinded to tissue type. The observers scored the nuclear and cytoplasmic immunostaining on a scale ranging from 0 (no immunostaining) to 3 (strong immunostaining) in each of 100 nuclei or cells, respectively, to yield a visual score for each observor ranging from 0 to 300 for each feature in each specimen as described by Miyamoto et al. [47]. The intensity of cytoplasmic immunostaining also was assessed by quantitative image analysis using Image Pro Plus software (MediaCybernetics, Bethesda, MD). A circular region of fixed area was used to extract the intensity information for cytoplasmic immunostaining of all epithelial cells in each image scored visually. Mean optical density (MOD) was derived from mean intensity (I_i) by setting the background intensity to white ($I_o = 255$) and using the equation [48]:

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$$MOD = -\log\left(\frac{I_i}{I_o}\right)$$

Mean visual scores and MOD were analyzed using Minitab (State College, PA). ANOVA (Tukey's honestly significant differences test) was used to compare visual scores of nuclear and cytoplasmic immunostaining and automated cytoplasmic immunostaining MOD [44]. Differences were considered statistically significant if $P < 0.05$.

RESULTS

Validation of the RPCI-5αR3 Monoclonal Antibody

Expression of 5α-reductase-3 at the mRNA level was analyzed in the BP epithelial cell line PWR-1E and the androgen-sensitive CaP cell lines, LNCaP, and LAPC-4, and the castration-recurrent CaP cell lines, C4-2 and CWR-R1 (Fig. 1A). 5α-reductase-3 mRNA expression in androgen-stimulated CaP and castration-recurrent CaP cell lines were expressed relative to PWR-1E cell line, which expressed very low levels of 5α-reductase-3 mRNA as determined using standard PCR. High levels of 5α-reductase-3 mRNA expression compared to PWR-1E were observed in the LNCaP, LAPC-4, and CWR-R1 cell lines. However, the castration-recurrent C4-2 cell line showed 5α-reductase-3 mRNA levels comparable to the PWR-1E cell line. These results were confirmed at the protein level using western blot analysis and the RPCI-5αR3 antibody (Fig. 1B). A single band for 5α-reductase-3 of in the range of 25–37 kDa was observed in total protein extracts of LAPC-4 and CWR-R1 cells (Fig. 1B), which corresponds to the expected size for 5α-reductase-3. However, no immuno-reactive band was observed in LNCaP cells, even though LNCaP expressed similar 5α-reductase-3 mRNA levels as LAPC-4 and CWR-R1. No immunoreactive band in the range of 25–37 kDa was observed in PWR-1E, C4-2, and CHO-K1 cell lines (Fig. 1B). CWR-R1 cells were chosen to compare sensitivity of the RPCI-5αR3 and SDR5A3 (Sigma) antibodies. No immuno-reactive band in the range of 25–37 kDa was detected using the SDR5A3 antibody, even when concentrations were 5-fold higher than suggested by the manufacturer (Fig. 1C). A weak band was appreciated using SDR5A3 antibody with longer times of exposure of the films (over 5 min). Both antibodies (RPCI-5αR3 and SDR5A3) recognized a single band over 50 kDa, which does not correspond to the expected size for 5α-reductase-3 (data not shown). Sub-cellular localization of 5α-reductase-3 protein and specificity of RPCI-5αR3 antibody were confirmed using immunostaining analyses. RPCI-5αR3 antibody showed a cytoplasmic immunostaining pattern (Fig. 1D); no nuclear immunostaining was observed. Incubation of RPCI-5αR3 antibody with the inhibitor peptide at increasing concentration (1 and 10 μg/ml) for 2 hr before immunostaining produced a peptide concentration-dependent inhibition of 5α-reductase-3 immunostaining in CWR-R1 cells (Fig. 1D). Complete inhibition of 5α-reductase-3 immunostaining was achieved at peptide concentration of 10 μg/ml.

5α-Reductase-3 Expression in Benign Human Tissues

Semi-quantitative analysis of 5α-reductase-3 expression was performed in a panel of human benign tissues, which included androgen-responsive (liver [n = 20], skeletal muscle [n = 2],

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skin [n = 1] testes [n = 20], thyroid [n = 20]), and androgen-insensitive tissues (brain [n = 20], breast [n = 20], colon [n = 20], endometrium [n = 20], kidney [n = 20], lung [n = 20], myometrium [n = 20], ovary [n = 20], pancreas [n = 15], spleen [n = 20], [n = 20], stomach [n = 20], and tonsil [n = 15]) (Figs. 2 and 3, Table I). High levels of 5α-reductase-3 protein expression were observed in a subset of human samples, which included skin (*stratum basale* and *stratum spinosum*), kidney (mostly proximal and some distal convoluted tubules), liver, skeletal muscle, myometrium, and pancreas (secretory epithelial cells). Moderate levels of expression of 5α-reductase-3 protein were observed in testis (Leydig cells), brain (neurons), breast (myoepithelial cells), colon (epithelial cells from colonic glands), and stomach (epithelial cells from the base of the gastric glands). Low levels of 5α-reductase-3 immunostaining were observed in lung (bronchial epithelium), and thyroid (cuboidal epithelium from thyroid follicles). 5α-reductase-3 was immunohistochemically undetectable in ovary, spleen, endometrium, and tonsil. In most cases, 5α-reductase-3 immunostaining was distributed homogeneously within the cytoplasm. However, perinuclear localization was observed when 5α-reductase-3 immunostaining was intense. 5α-reductase-3 protein expression was observed both in conventional androgen-responsive benign tissues, such as skin and skeletal muscle, and non-conventional androgen-responsive tissues, such as kidney, colon and pancreas.

5α-Reductase-3 Expressionin Malignant Human Tissues

As was observed in benign tissues, 5α-reductase-3 immunostaining in malignant tissues showed inter-organ and inter-patient variability (Figs. 4 and 5, Table II). High levels of 5α-reductase-3 immunostaining were observed in malignant tissues from kidney (chromophobe carcinoma), liver (hepatocellular carcinoma), stomach (adenocarcinoma), thyroid (papillary carcinoma), colon (adenocarcinoma), and uterus (endometrioid adenocarcinoma). Moderate to low levels of 5α-reductase-3 immunostaining were observed in adrenal (pheochromocytoma), bladder (high grade urothelial carcinoma), breast (lobular and metaplastic carcinomas), esophagus (adenocarcinoma), kidney (clear cell renal cell carcinoma and papillary carcinoma), lung (adenocarcinoma), ovary (mucinous adenocarcinoma), testis (seminoma, embryonal and yolk sac carcinomas), and thyroid (medullary carcinoma). 5α-reductase-3 was immunocytochemically undetectable in bladder (low grade urothelial carcinoma and small cell carcinoma), lung (mesothelioma), leiomyosarcoma, and ovary (serous adenocarcinoma). 5α-reductase-3 immunostaining was confined to the cytoplasm of malignant epithelial cells, which exhibited perinuclear localization when intense. Among the malignant human tissues analyzed, only breast (lobular and metaplastic carcinomas), testis (seminoma, embryonal, and yolk sac carcinomas), lung (adenocarcinoma), and thyroid (papillary carcinoma) clearly showed over-expression of 5α-reductase-3 protein compared to their benign counterpart (compare Tables I and II). However, in the majority of cases, similar levels of 5α-reductase-3 immunostaining were observed between the benign and malignant tissue pairs (compare Tables I and II).

5α-Reductase Expression During Carcinogenesis and Progression of Prostate Cancer

5α-Reductase-3 immunostaining was analyzed in clinical specimens of different types of prostate tissues, since the prostate is a highly androgen-responsive organ (Fig. 6). In BP, 5α-

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reductase-3 immunostaining was observed primarily at the periphery of the benign glands, which suggests 5α-reductase-3 expression in the basal cell compartment. This observation was confirmed by co-localization of 5α-reductase-3 and p63, a basal cell marker. Low to undetectable levels of 5α-reductase-3 immunostaining were observed in luminal epithelial cells. In HGPIN, 5α-reductase-3 immunostaining localized to both basal cells and neoplastic luminal epithelial cells. However, 5α-reductase-3 was low in luminal epithelial cells of adjacent benign glands. In androgen-stimulated CaP and castration-recurrent CaP, 5α-reductase-3 immunostaining was found in the cytoplasm of most malignant epithelial cells. 5α-reductase-3 immunostaining was mostly perinuclear when intense. Cytoplasmic 5α-reductase-3 immunostaining intensity was similar in androgen-stimulated CaP and castration-recurrent CaP, and was higher than androgen-stimulated BP, when compared using visual scores (ANOVA, $P < 0.00001$) or image analysis (ANOVA, $P < 0.00001$) (Table III). Nuclear immunostaining was minimal and varied marginally among androgen-stimulated BP, androgen-stimulated CaP, and castration-recurrent CaP (ANOVA, $P = 0.205$) (Table III). 5α-reductase-3 immunostaining in malignant epithelial cells was confirmed by co-localization of 5α-reductase-3 and AMACR immunostaining.

DISCUSSION

Several pieces of evidence suggested the presence of a third isozyme of 5α-reductase: a candidate gene sequence [31], low response rate of men with castration-recurrent CaP to dutasteride in a clinical trial [49], and failure of bi-specific 5α-reductase inhibitors to eliminate tissue levels of DHT (dutasteride reduced tissue levels of DHT by 94% in androgen-stimulated BP [50] and LY320236 failed to decrease serum DHT levels in intact men [51]). Uemura et al. [32] confirmed the existence of a novel 5 alpha-steroid reductase (SRD5A3, type-3) in human tissues but only at the mRNA level. They reported that 5α-reductase-3 was overexpressed in castration-recurrent CaP cells. Gaining insight into the localization and function of 5α-reductase-3 has proven difficult because commercially available antibodies against 5α-reductase-3 became available only recently and characterizing 5α-reductase-3 enzymatic activities is challenging, because the enzymes appear embedded within the endoplasmic reticulum, are hydrophobic, and form inclusion bodies when expressed *in vitro*. Our laboratory produced a highly specific monoclonal antibody (RPCI-5αR3) against the amino-terminal portion of the 5α-reductase-3 protein and analyzed expression of this protein in benign and malignant human tissues, with special emphasis on prostate carcinogenesis and CaP progression. During the course of these studies, a commercially available polyclonal antibody against 5α-reductase-3 was released from Sigma. The Sigma antibody at concentrations that ranged from 1 μg/ml (concentration suggested by the manufacturer) to 5 μg/ml revealed no immuno-reactivity using western blot. The RPCI-5αR3 antibody, however, detected 5α-reductase-3 protein using immunocytochemistry, immunohistochemistry, and western blot. Further analyses are required to validate the RPCI-5αR3 antibody for ELISA and immunoprecipitation.

5α-Reductase-3 expression analyses in human tissues performed by Uemura et al. [32] were based on northern blot analysis. Their tissue expression analyses revealed very low levels of expression of 5α-reductase-3 mRNA in most benign adult organs, with benign pancreas showing the highest levels of expression of 5α-reductase-3 mRNA. These results, however,

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contrast with the data reported by Yamana et al. [52] using QRT-PCR, which indicated a broader and higher expression of 5α-reductase-3 mRNA compared to 5α-reductase-1 and 2 mRNAs in human tissues. Our immunostaining analyses support Yamana's results and showed variable levels of expression of 5α-reductase-3 protein in several benign human tissues. The differences in expression of 5α-reductase-3 at the mRNA and protein levels between Uemura's study and our immunostaining analysis could be explained, at least in part, by differences in the sensitivity of the techniques utilized (northern blot versus immunostaining). Alternatively, presence of mRNA does not always correlate with expression of protein, as was demonstrated clearly in this study for 5α-reductase-3 expression in LNCaP cells, which highlights the importance of our immunostaining analysis.

5α-Reductase-3 protein was expressed in both conventional androgen-regulated human tissues, such as skeletal muscle, skin, and prostate, and in non-conventional androgen-regulated tissues, such as pancreas, colon, and kidney. These data suggest either multiple target tissues for androgens or different functions of 5α-reductase-3 among benign human tissues. In support of the second hypothesis, Cantagrel et al. [53], reported that mutations in 5α-reductase-3 gene caused congenital disorders of glycosylation, which induce mental retardation and ophthalmologic and cerebellar defects. In this study, 5α-reductase-3 protein was found necessary for the reduction of the α-isoprene unit of polyprenols to form dolichols, which are required for the N-glycosylation process. Unpublished data from our laboratory using protein assays of recombinant 5α-reductase-3 expressed in CHO cells and bacteria suggest conversion of T, androstenedione, and progesterone to DHT, androstanedione, and 5α-pregnan-3-20-dione, respectively. Broad substrate specificity of this enzyme is consistent with ubiquitous expression of 5α-reductase-3 protein in human tissues.

In tumor tissues, 5α-reductase-3 protein expression was observed in several different types of malignancies. Among those tissues that expressed moderate to high levels of 5α-reductase-3, only cancers of breast, testis, lung, thyroid, and prostate showed overexpression of 5α-reductase-3 compared to their benign counterpart. Neither 5α-reductase-1 nor 5α-reductase-2 has been advocated as a potential biomarker of malignancy. However, Uemura et al. [32] suggested over-expression of 5α-reductase-3 at the mRNA level in castration-recurrent CaP compared to BP. Even though the number of clinical specimens analyzed in this study is limited, these results warrant further investigation of a putative role for 5α-reductase-3 as a biomarker of malignancy in the prostate, breast, testis, lung, and thyroid tissues.

Analysis of archival specimens suggested that 5α-reductase-3 may provide a biomarker of malignancy in the prostate. 5α-reductase-3 was expressed in the basal cells of androgen-stimulated BP but was not detected in the benign luminal epithelium. In contrast, in CaP that lacks basal cells, 5α-reductase-3 was found in the malignant epithelium. HGPIN is hypothesized to be the precursor lesion for CaP [54]. In all six specimens of HGPIN examined, 5α-reductase-3 was present in the basal cell layer of both benign glands and HGPIN, and although 5α-reductase-3 was not detected in luminal epithelial cells in benign glands adjacent to HGPIN lesions, 5α-reductase-3 was expressed in the neoplastic cells of HGPIN lesions. These findings suggest that a characteristic of the development of CaP may

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be the transition of 5α-reductase-3 expression from the basal cell compartment to the cancer epithelial cell compartment; maintenance of 5α-reductase-3 expression may be a characteristic of progression.

We [44] and others [55] reported previously that 5α-reducing capacity shifted from 5α-reductase-2 in androgen-stimulated BP to 5α-reductase-1 in androgen-stimulated CaP and castration-recurrent CaP. However, some if not most, of the 5α-reducing capability of castration-recurrent CaP may be due to 5α-reductase-3. However, the presence of high levels of 5α-reductase-3 protein, and new knowledge gained of the potential importance of 5α-reduction in preclinical models of castration-recurrent CaP [29,30,56] suggest an important role for 5α-reductase-3 in the lethal phenotype of CaP. The potential clinical importance of 5α-reductase-3 in CaP requires further investigation but tri-specific 5α-reductase-3 inhibitors may prove useful for treatment of benign prostatic enlargement and prevention and treatment of CaP, especially advanced CaP. These common diseases all depend on tissue levels of DHT for AR transactivation that may persist in spite of currently available treatments due to unblocked 5α-reductase-3 activity.

CONCLUSIONS

The results of this study allow one to conclude that: (i) expression of 5α-reductase-3 in “classical” as well as “non-classical” androgen-regulated tissues is consistent with 5α-reductase-3 enzyme having functions other than converting T to DHT in human tissues, such as participation in the N-glycosylation process; (ii) over-expression of 5α-reductase-3 in breast, testis, lung, thyroid, and particularly prostate cancer, compared to their benign counterparts, suggests a potential role for 5α-reductase-3 as a biomarker of malignancy; and (iii) over-expression of 5α-reductase-3 in AS-CaP and CR-CaP suggests a potential role for this enzyme in synthesizing DHT in both an androgen-stimulated and an androgen-deprived human prostate microenvironment.

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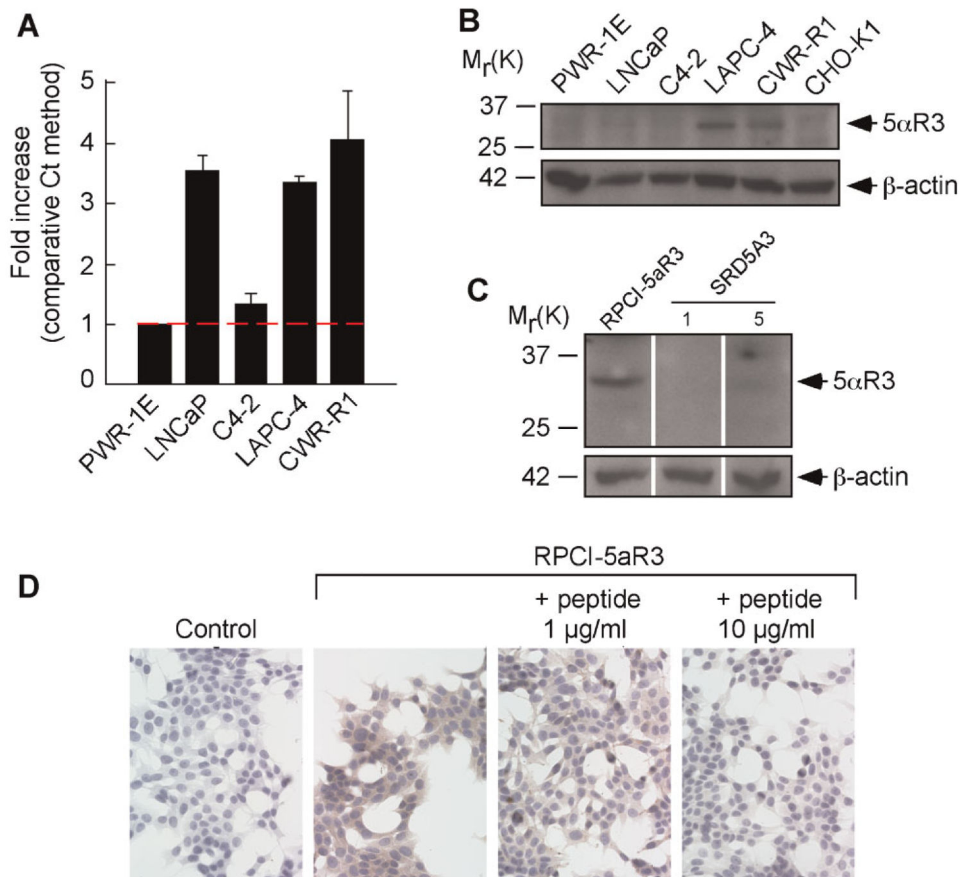

Fig. 1.

Validation of the RPCI-5αR3 antibody. **A**: 5α-reductase-3 mRNA expression level in LNCaP, C4-2, LAPC-4, and CWR-R1 cells. 5α-reductase-3 mRNA expression levels in the CaP cell lines were normalized to the level of expression of 5α-reductase-3 in the PWR-1E benign prostate epithelial cell line (discontinuous red line). **B**: PWR-1E, LNCaP, C4-2, LAPC-4, CWR-R1, and CHO-K1 protein lysates (50 μg) were immunoblotted and 5α-reductase-3 protein expression was detected using the RPCI-5αR3 antibody. **C**: CWR-R1 lysates (50 μg) were immunoblotted and 5α-reductase-3 protein expression was analyzed using RPCI-5αR3 antibody at a concentration of 1 μg/ml or SRD5A3 (Sigma) antibody at concentrations 1 μg/ml and 5 μg/ml. **D**: Immunostaining analyses were performed in CWR-R1 using the RPCI-5αR3 antibody. Preincubation of the RPCI-5αR3 antibody with the inhibitor peptide (1 and 10 μg/ml) confirmed specificity. Absence of primary antibody provided negative control (Control).

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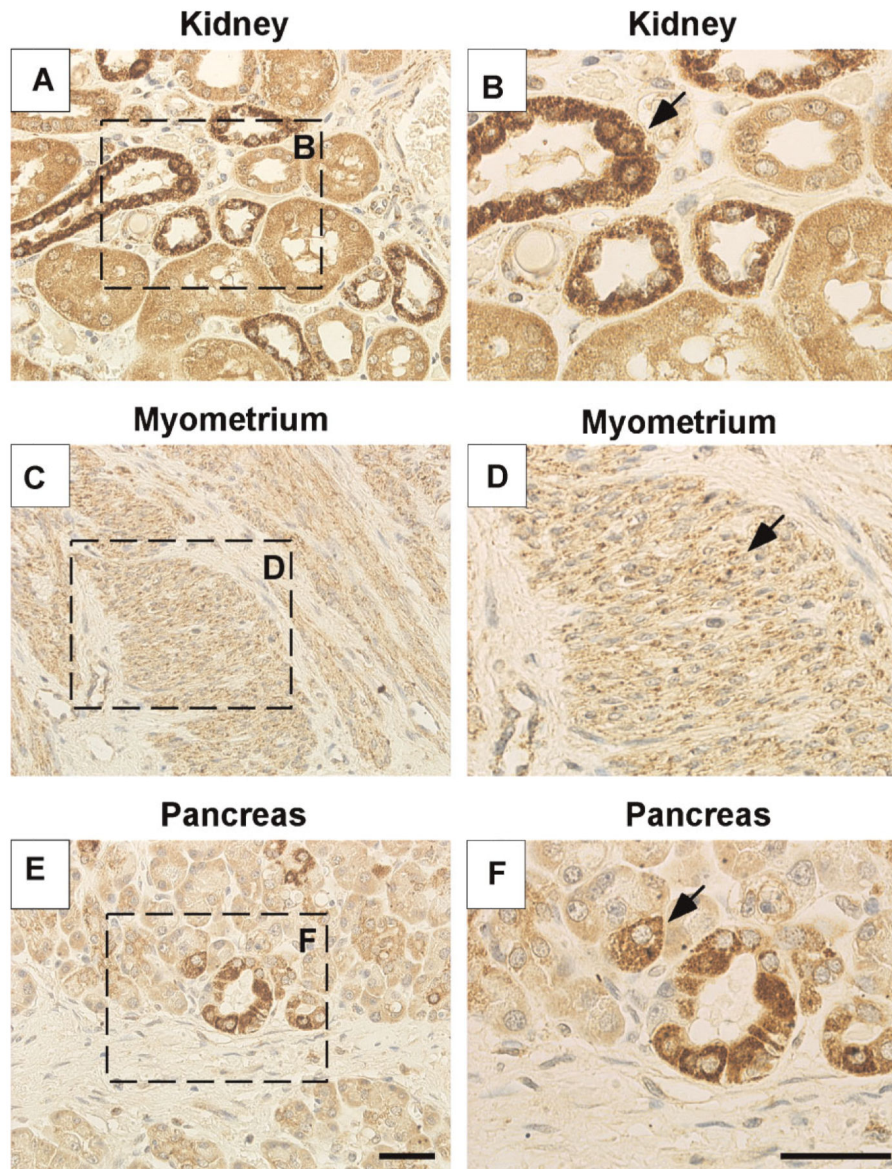

Fig. 2.
5α-reductase-3 immunostaining in benign human tissues. High level expressing tissues (representative sections that demonstrate semi-quantitative data in Table I). In kidney **A,B**: 5α-reductase-3 expression was localized to the cytoplasm of the epithelial cells from some of the proximal and distal convoluted tubules (**B**, arrow). In myometrium **C,D**: 5α-reductase-3 immunostaining was located preferentially to the cytoplasm of the smooth muscle cells (**D**: arrow, fibers in transverse). In pancreas **E,F**: 5α-reductase-3 immunostaining was observed in some of the secretory cells from the acini (**F**, arrow). Bars: 30 μm.

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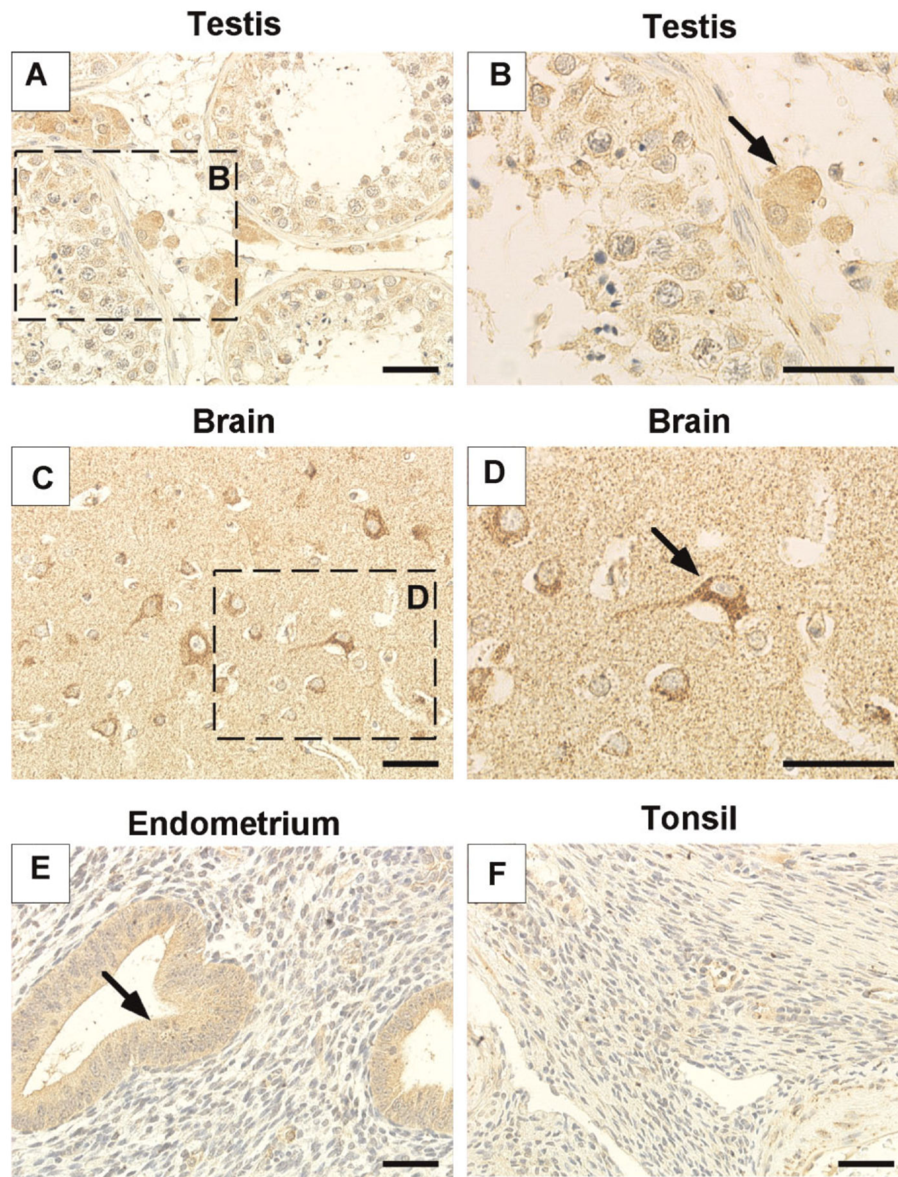

Fig. 3.
5α-reductase-3 immunostaining in benign human tissues. Moderate to low level expressing tissues (representative sections that demonstrate semi-quantitative data in Table I). In testes **A,B**: 5α-reductase-3 expression was localized to the cytoplasm of Leydig cells (**B**, arrow). In brain **C**: 5α-reductase-3 immunostaining was localized to the cytoplasm of neurons (**D**, arrow). Low levels of 5α-reductase-3 immunostaining were observed in the glandular epithelium of endometrium (**E**, arrow). 5α-reductase-3 was immunohistochemically undetectable in tonsil (**F**). Bars: 30 μm.

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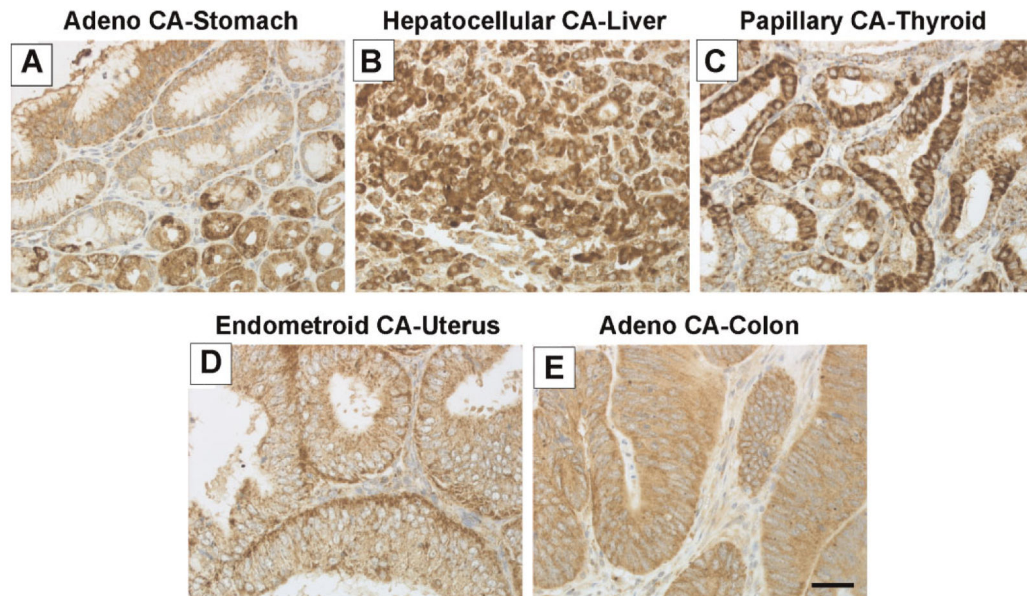

Fig. 4.

5α-reductase-3 immunostaining in malignant human tissues. High level expressing tissues (representative sections that demonstrate semi-quantitative data in Table II). 5α-reductase-3 expression was localized mainly to the cytoplasm of the malignant epithelial cells from adenocarcinoma of the stomach (**A**), hepatocellular carcinoma of the liver (**B**), papillary carcinoma of the thyroid (**C**), endometroid adenocarcinoma of the uterus (**D**), and adenocarcinoma of the colon (**E**). Bar: 30 μm.

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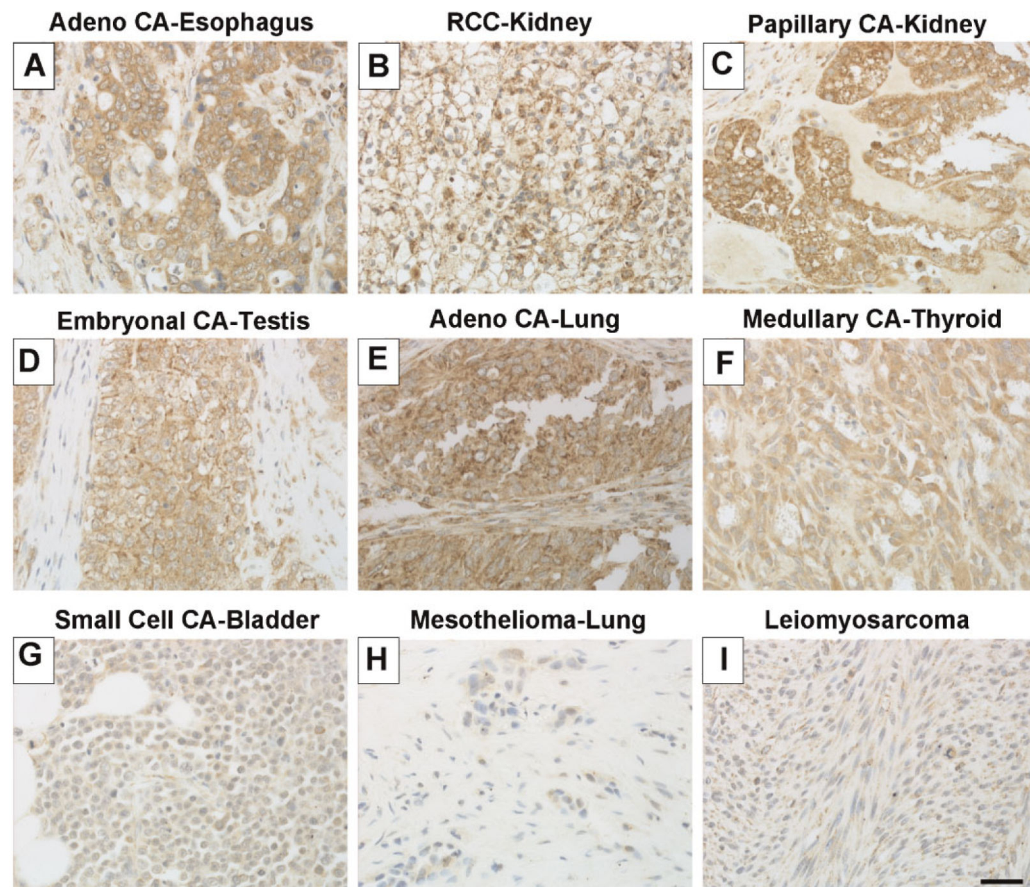

Fig. 5.

5α-reductase-3 immunostaining in malignant human tissues. Moderate to low level expressing tissues (representative sections that demonstrate semi-quantitative data in Table II). Moderate to low levels of 5α-reductase-3 expression were observed in the cytoplasm of neoplastic epithelial cells from adenocarcinoma of the esophagus (**A**), clear cell renal cell carcinoma of the kidney (**B**), papillary carcinoma of the kidney (**C**), embryonal testicular carcinoma (**D**), adenocarcinoma of the lung (**E**), and medullary carcinoma of the thyroid (**F**). Negative 5α-reductase-3 immunostaining was observed in small cell carcinoma of the bladder (**G**), mesothelioma (**H**), and leiomyosarcoma (**I**). Bar: 30 μm.

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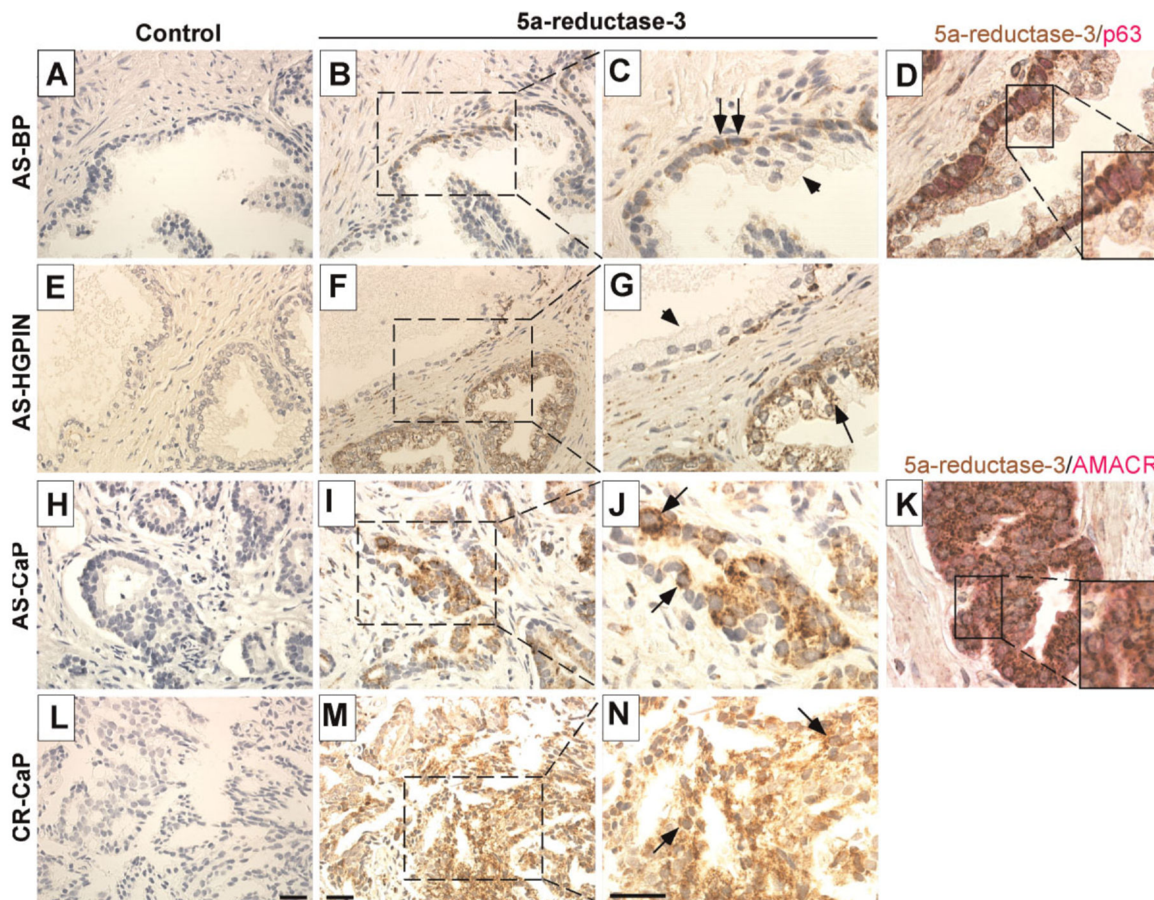

Fig. 6.

5α-reductase-3 immunostaining in androgen-stimulated benign prostate (AS-BP), androgen-stimulated high grade intraepithelial neoplasia (AS-HGPIN), androgen-stimulated CaP (AS-CaP), and castration-recurrent CaP (CR-CaP) tissue sections (representative sections that demonstrate quantitative data in Table III). 5α-reductase-3 immunostaining in AS-BP was observed primarily at the periphery of benign glands, which suggests 5α-reductase-3 expression in the basal cell compartment (B, C [arrows]) that was confirmed by co-localization of 5α-reductase-3 (brown cytoplasmic stain) and p63 (rednuclear stain) immunostaining (**D**). No to low levels of 5α-reductase-3 immunostaining were observed in benign luminal epithelial cells (**B** [arrowhead]). In HGPIN, 5α-reductase-3 immunostaining was located in both basal cells and hyperproliferative malignant luminal epithelial cells (**F,G** [arrow]) but no to low levels of 5α-reductase-3 immunostaining were observed in luminal epithelial cells of adjacent benign glands (**F,G** [arrowhead]). In AS-CaP and CR-CaP, 5α-reductase-3 immunostaining was located in most malignant epithelial cells (**I,J,N**). 5α-reductase-3 immunostaining was mostly perinuclear when intense (**J,N** [arrows]. 5α-reductase-3 immunostaining in malignant epithelial cells was confirmed by co-localization of 5α-reductase-3 (brown cytoplasmic stain) and AMACR (red cytoplasmic stain) immunostaining (**K**). Incubation in the absence of primary antibody provided negative

controls (**A,E,H,L**). Black bars: 30 μM. Images reduced from 200× magnification (columns 1 and 2) or 400× magnification (columns 3 and 4).

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TABLE I

5α-Reductase-3 Immunostaining in Benign Human Tissues

Tissue	Number of positive cases/ total cases	Staining intensity	Preferential localization
Brain	6/20	−/+	Neurons
Breast	4/20	−/+	Myoepithelial cells
Colon	7/20	−/++	Epithelial cells from colonic glands
Endometrium	10/20	−/+	Glandular epithelium
Kidney	20/20	+++	Epithelial cells (PCT, DCT)
Liver	20/20	+++	Hepatocytes
Lung	2/20	−/+	Bronchial epithelium
Myometrium	11/20	++	Smooth muscle cells
Ovary	0/20	−	
Pancreas	15/15	++/+++	Secretory cells (GA)
Prostate	23/25	++	Basal cells
Skeletal muscle	2/2	+/++	Skeletal muscle cells
Skin	1/1	+/++	*Stratum basale, stratum spinosum*
Spleen	0/20	−	
Stomach	12/20	+/++	Epithelial cells from the base of the gastric glands
Testes	8/20	−/+	Leydig cells
Thyroid	13/20	−/+	Cuboidal epithelium from thyroid follicles
Tonsil	0/15	−	

Criteria for immunohistochemical analysis: −, negative staining; +, weak staining; ++, moderate staining; +++, intense staining; PCT, proximal convoluted tubule; DCT, distal convoluted tubule; GA, glandular acini.

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TABLE II
5α-Reductase-3 Immunostaining in Malignant Human Tissues

Tissue	Number of positive cases/ total cases	Staining intensity
Adrenal Pheochromocytoma	1/2	−/+
Bladder		
Low grade TCC	0/2	−
High grade TCC	2/2	+
Small Cell CA	2/2	−
Breast		
Lobular CA	2/2	+
Metaplastic CA	2/2	+
Colon Adeno CA	2/2	++
Esophagus Adeno CA	2/2	+/++
Kidney		
Chromophobe CA	4/4	+++
Papillary CA	1/2	++
Renal Clear Cell CA	2/2	++
Liver HCC	2/2	++/+++
Lung		
Adeno CA	2/2	++
Mesothelioma	2/2	−
Leiomyosarcoma	2/2	−
Ovary		
Mucinous CA	4/4	+/++
Serous CA	0/2	−
Stomach Adeno CA	2/2	++/+++
Testis		
Seminoma	2/2	++
Embryonal CA	2/2	+
Yolk Sac CA	1/2	++
Thyroid		
Papillary CA	4/4	+++
Medullary CA	2/2	+
Uterus Endometroid CA	2/2	++

Criteria for immunohistochemical analysis: −, negative staining; +, weak staining; ++, moderate staining; +++, intense staining; TCC, transitional cell carcinoma; CA, carcinoma; HCC, hepatocellular carcinoma.

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TABLE III
5α-Reductase-3 Immunostaining of AS-BP, AS-CaP, and CR-CaP

	Mean ± SE		
	AS-BP	**AS-CaP**	**CR-CaP**
Visual score			
Nuclear	14.7 ± 4.4	25.6 ± 3.5	29.2 ± 8.4
Cytoplasmic	115.6 ± 10.5	200.5 ± 9.1[a]	163 ± 11.7[a]
MOD			
Cytoplasmic	0.32 ± 0.02	0.50 ± 0.03[a]	0.49 ± 0.03[a]

AS-BP, androgen-stimulated BP; AS-CaP, androgen-stimulated CaP; CR-CaP, castration-recurrent CaP; SE, standard error.

[a]Statistically different than androgen-stimulated BP.