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Variability in the androgen response of prostate epithelium to 5-alpha reductase inhibition: implications for prostate cancer chemoprevention

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Abstract

Androgens and the androgen receptor (AR) influence prostate carcinogenesis. Lowering intraprostatic dihydrotestosterone (DHT) by inhibiting 5-alpha-reductase (SRD5A) reduces prostate cancer (PCa) incidence, but is not uniformly effective. Mechanisms by which SRD5A inhibition influences PCa initiation and/or progression among different individuals have not been established. We sought to identify molecular alterations underlying the differential chemo-preventive activity of SRD5A inhibition. Men with clinically-localized PCa were randomized to prostatectomy alone (n=25) or 4 months treatment with the SRD5A-inhibitor dutasteride (0.5mg (n=26) or 3.5mg (n=24)) preceding prostatectomy. Serum and prostate androgens were measured using mass spectrometry. We evaluated benign epithelial gene expression using expression profiling and immunohistochemistry, and characterized tumor TMPRSS2-ERG fusion status using FISH. Dutasteride at 0.5 or 3.5mg decreased prostatic DHT by 93% (0.23ng/g; $p<0.001$) and 98.8% (0.04ng/g; $p<0.001$) vs. untreated patients (3.33ng/g). Despite significant and uniform suppression of tissue DHT, unsupervised clustering based on prostatic gene expression did not allow us to discriminate dutasteride-treated from untreated individuals. However, we could resolve subjects into distinct cohorts characterized by high or low expression of AR-regulated genes (irrespective of treatment dose) based solely on AR transcript expression. The high-dose dutasteride treatment group comprised significantly fewer cancers with TMPRSS2-ERG fusions. Despite substantial and uniform reductions in prostatic DHT, dutasteride was associated with highly variable alterations in benign epithelial gene expression. Segregation of subjects based on AR and androgen-regulated gene expression indicates patients are differentially sensitive to SRD5A inhibition. Tissue AR levels may serve as a pretreatment predictor of SRD5A chemo-preventive efficacy.

Keywords

prostate cancer; chemo-prevention; 5-alpha-reductase; androgen receptor; dutasteride

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INTRODUCTION

Androgens and the androgen receptor (AR) regulate prostate development and carcinogenesis. (1,2) Within the prostate, testosterone (T) is converted to the more potent androgen, dihydrotestosterone (DHT) by 5a-reductase (SRD5A) types 1 and 2, and possibly type 3. (3, 4) The importance of SRD5A in mediating prostate carcinogenesis was tested in the Prostate Cancer Prevention Trial (PCPT) where the SRD5A2 inhibitor finasteride decreased prostate cancer (PCa) incidence 25% (18% vs. 24% in placebo-treated patients). (5) This striking benefit was tempered by an increase in high-grade cancers, subsequently attributed to differential effects of finasteride on prostate volume and a possible selective inhibition of low-grade cancers. (6) Using the dual SRD5A inhibitor dutasteride, the REduction by Dutasteride of Prostate Cancer (REDUCE) study reported a similar 23% reduction in PCa incidence (low-grade cancers in 13.3% vs. 18.1% of placebo-treated patients ($p<0.0001$)), with no difference in high-grade cancers. (7)

Mechanisms by which SRD5A inhibition decreases PCa incidence have not been determined, but likely reflect decreased AR-axis activity caused by reduced tissue DHT, potentially resulting in regression of extant cancers or suppression of *de novo* tumorigenesis. Importantly, 16–18% of subjects in both PCPT and REDUCE were diagnosed with PCa(of low and high-grade histologies) despite undergoing SRD5A inhibition. Factors responsible for the variable outcome of SRD5A inhibition in reducing PCa incidence among different individuals are unknown.

We sought to identify dutasteride-related molecular alterations underlying the chemo-preventive activity of SRD5A inhibition, and to identify the impact of SRD5A inhibition on the prostatic AR axis. We evaluated prostate tissues from a randomized study of 81 men with clinically-localized PCa undergoing surgery alone or after 4 months dutasteride treatment. (8) We quantitated gene expression changes in micro-dissected prostate epithelium, and evaluated gene activity in relation to intra-prostatic T and DHT concentrations.

MATERIALS AND METHODS

Clinical Protocol

We obtained prostate tissue samples from a clinical trial evaluating dutasteride (Dut) (GlaxoSmithKline, Research Triangle Park, NC) prior to radical prostatectomy (RP) as described. (8) Procedures involving human subjects were approved by Institutional Review Boards of participating institutions; all subjects signed written informed consent. Tissues obtained at prostatectomy were frozen in Optimal Cutting Temperature (Tissue Tek OCT, Sakura-Finetek, Torrance, CA) and formalin-fixed and paraffin-embedded (FFPE). During the original clinical study, resected cancerous prostate tissues were preferentially placed in formalin for paraffin embedding. Histologic analysis of multiple sections from provided frozen tissue blocks revealed malignant prostate glands in <5% of samples; thus, we did not evaluate mRNA levels in malignant prostate epithelium.

Serum and Prostatic Androgen Measurements

Serum and prostate androgen levels were determined using gas chromatography/mass spectroscopy as described (PPD Development, Richmond, Virginia). (8) Raw data for individual subjects (previously published in aggregate (8)) were utilized for present analyses.

Gene Expression Assays

Laser Capture Microdissection (LCM), RNA Amplification and Microarray Hybridization—We used frozen prostate samples for LCM of benign tissue (neoplastic tissue

was not identified in a sufficient number of frozen samples to allow analysis). We collected 2000-3000 benign epithelial cells per sample using the Arcturus Veritas™ LCM System (Mountain View, CA) for isolation of total RNA followed by two rounds of linear amplification as described. (1,9) Probe pairs (2 μg amplified RNA from microdissected samples and 30 μg total RNA from a reference prostate cell-line RNA pool) were hybridized to custom cDNA microarrays as described. (1,9) Fluorescence images were collected (GenePix 4000B, Axon Instruments, Foster City, CA) and processed as described. (10) We evaluated expression changes using two sample t tests (Statistical Analysis of Microarray (SAM) (http://www-stat.stanford.edu/_tibs/SAM/)). (11) FDR (false discovery rate) less than 5% was considered significant. Microarray data can be accessed via the GEO database (GSM251831).

Cluster Analysis—We performed unsupervised hierarchical average linkage clustering using Cluster 3.0 software (http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/software.htm) plotted using TreeView version 1.6 (http://rana.lbl.gov/EisenSoftware.htm). We clustered samples using the top 1000 most variable genes from microarray expression profiling (those with highest inter-quartile ratio (IQR), representing the spread between the 75^{th} and 25^{th} percentile of expression data obtained for each gene), or using a custom list of 90 androgen-regulated genes (see below).

Generation of androgen-regulated gene list for clustering—We included genes in the androgen-regulated set if they were present in at least two of the following sources: NetPath Androgen Receptor Pathway (http://www.netpath.org/), a transcript profiling study of androgen-mediated expression in LNCaP cells by Nelson, et al. (1), a transcript profiling study of androgen-mediated expression in multiple prostate cell lines by DePrimo et al. (12) (≥ 3 fold change), and results from a whole genome Agilent microarray comparing LNCaP and VCaP with or without the synthetic androgen R1881 (Mostaghel and Nelson, unpublished results) (≥ 3 fold change)). The 90 genes generated by this analysis are presented in Supplementary Data (Supp) 1.

Quantitative RT-PCR—We validated gene expression changes using qRT-PCR in triplicate reactions using 5 ng of cDNA, 1 μM of each primer pair and SYBR-Green PCR master mix (Applied Biosystems, Foster City, CA). We normalized mean cycle threshold (Ct) for each gene to a housekeeping gene, RPL13A, in the same sample using the delta CT method. (Primer sequences in Supp. 2).

Analysis of AR transcript expression in human prostate samples—We used four human prostate microarray datasets to compare AR expression in benign prostate tissue. These consisted of 40 benign prostate samples adjacent to cancer analyzed on spotted 44K human cDNA microarrays by Lapointe et al. (13); 50 benign prostate samples adjacent to cancer analyzed on U95Av2 human Affymetrix (Santa Clara, CA) arrays by Singh et al. (14); 23 benign prostate samples from normal donors plus 63 benign prostate samples adjacent to cancer analyzed on Affymetrix U95a, U95b, and U95c chip sets by Yu et al. (15); and 11 benign prostate samples on U95Av2 human Affymetrix arrays analyzed by Glinsky et al. (16) All studies used bulk tissue. We analyzed two human prostate sample sets by qRT-PCR for AR expression in microdissected benign prostate epithelium: the untreated samples obtained from the dutasteride treatment study reported here and benign prostate epithelium in prostate biopsy cores from 10 untreated individuals.

Immunohistochemistry AND FISH

Tissue Microarray (TMA) and Immunohistochemistry (IHC)—A TMA comprising benign and cancer tissue cores from each patient (n=81) was generously provided by M. Gleave. Benign and cancer sites were each sampled 3 times (0.6mm diameter) creating a triplet

TMA layout (426 cores total). 0.5 micrometer sections were mounted on charged slides for staining with TMPRSS2 (previously described (17)) and TFF3 (ab57752, 3ug/ml, Abcam, Inc). We individually scored staining in each core on a 4-point scale from none (0) to high (3). We compared staining intensity in control and treatment groups by fitting a logistic regression model using generalized estimating equations to account for multiple observations per patient; p values <0.05 were considered significant.

Fluorescence In-Situ Hybridization (FISH)—We performed FISH analysis for TMPRSS2-ERG chromosomal fusion on the TMA using published probes and methods. (18) No cores without carcinoma exhibited a TMPRSS2-ERG fusion. We tabulated fusion status for TMA cores containing cancer blinded to treatment group. We determined significance between treatment groups using a logistic regression model ($\log(p/(1-p))=B_0+B_1x_1$ where p denotes probability of fusion and x_1 denotes dutasteride dose) and fit using generalized estimating equations to account for multiple cores as well as different numbers of observations per subject due to loss of cores or absence of cancer.

RESULTS

Serum and prostatic androgen concentrations after dutasteride treatment

To evaluate molecular effects of SRD5A inhibition *in vivo*, we studied prostate tissues from a clinical trial of dutasteride prior to radical prostatectomy (RP). (8) Eighty-one men aged 45–80 years with localized PCa (T1c-T2b), Gleason score ≤7, and PSA 2.5-10 ng/dL were randomized to immediate RP (n=25) or four months of dutasteride (Dut) 0.5 mg (n=26) or 3.5 mg (n=24) orally daily preceding RP.

The proposed mechanism of SRD5A inhibition in PCa chemo-prevention is based on an overall reduction in prostate tissue androgens and attendant decrement in AR activity. Accordingly, dutasteride-treated subjects had significantly lower serum and tissue DHT levels than untreated men (Table 1). (8) Compared to untreated samples (3.33 ng/g), prostatic DHT was 93% and 98.8% lower in 0.5mg (0.23 ng/g) and 3.5mg (0.04 ng/g) groups, respectively. We observed reciprocal increases in tissue T due to inhibition of SRD5A activity in dutasteride-treated samples. Utilizing a conservative estimate that relative potency of DHT in engaging and maintaining AR activity is twice that of T (19), a ‘tissue androgen index’ (sum of T plus 2x DHT) suggests overall tissue androgen activity in dutasteride-treated samples is reduced by approximately 40–60% (Table 1). Using less conservative DHT to T potency ratios of 5:1 or 10:1 resulted in a 70–80% and 80–90% difference in estimated tissue androgen activity between untreated and dutasteride-treated samples (Table 1).

Dutasteride induces differential expression of androgen regulated genes in benign prostate epithelium

To assess effects of SRD5A inhibition on prostate gene expression, we microdissected benign epithelium from untreated and dutasteride-treated cohorts and evaluated transcript expression using cDNA microarrays. Unsupervised hierarchical clustering using all genes did not discriminate treated and untreated samples (data not shown). Clustering based on the 1000 most variably expressed genes similarly failed to distinguish surgery alone and dutasteride-treated groups (Figure 1a), and failed to resolve high and low dutasteride cohorts from one another (Figure 1b). These findings suggest the overall effects of dutasteride on prostate gene expression were modest and/or heterogeneous, and therefore unable to overcome the substantial variability in global transcript expression observed in untreated prostate epithelium.

However, direct comparison of prostatic gene expression in untreated and dutasteride-treated samples using two sample t-tests did reveal significant dutasteride-related alterations in

epithelial gene expression, with 120 genes ≥ 1.5 fold differentially regulated (FDR <5%) (Figure 2a and S.D. 3). Among these, we observed significant alterations in expression of known androgen-regulated genes, including up-regulation of IGFBP3 (3.7-fold), and down-regulation of TMPRSS2, KLK3 (PSA), KLK2, FKBP5, and KLK4 (1.8, 1.8, 2.0, 2.1 and 2.6-fold, respectively), confirmed by qRT-PCR (Figure 2b).

Variability in the molecular response to SRD5A inhibition distinguishes patients with high and low androgen responsive programs

A primary measure of the tissue response to dutasteride should be an effect on the cellular androgen axis, as reflected by tissue AR activity. Although expression profiling identified significant dutasteride-related differences in expression of many androgen-regulated genes, substantial heterogeneity among individuals was evident (Figure 2b). Many dutasteride–treated samples expressed transcripts for androgen-regulated genes within ranges measured in untreated samples and unrelated to treatment dose (Figure 2b). Moreover, despite significantly lower DHT levels in the 3.5 mg than 0.5 mg cohort (Table 1; 0.04 (+/− 0.02) ng/g vs 0.23 (+/− 0.12) ng/g; $p < 0.0001$), we did not identify differentially regulated genes between the 3.5 mg vs 0.5 mg dutasteride-treated groups (data not shown). These results suggest AR-mediated transcription reflects the total tissue androgen state and not absolute tissue DHT concentration, such that lower tissue DHT in the 3.5 mg group may potentially be attenuated by increases in tissue T. This conclusion is consistent with relatively similar overall estimates of tissue androgen indices in the two dutasteride-treated cohorts (Table 1).

To more specifically interrogate the impact of SRD5A inhibition on the prostatic AR axis, we evaluated dutasteride-treated samples for expression of 90 androgen-regulated genes (Supp. 1). (1,12) Unsupervised hierarchical clustering based on expression of these androgen-regulated genes resolved dutasteride-treated samples into two groups distinguished by markedly different expression levels of genes known to be highly responsive to androgen (FOLH1, NKX3-1, TMPRSS2, KLK2, KLK3, KLK4, and PPAP2A) (Figure 3a), as well as expression of the AR itself. We designated these cohorts AR Gene activity-High (ARG-Hi) and AR Gene activity-Low (ARG-Lo). Unsupervised clustering of the entire sample set (including untreated cases) using the same 90 androgen-regulated genes provided a near-identical segregation of treated samples, and placed 7 of 11 untreated samples into the ARG-Hi group (Figure 3b). Of interest in context of subsequent analyses, 3 of 4 untreated samples falling into the ARG-Lo group were among untreated samples with the lowest AR mRNA expression.

Variation in tissue response to dutasteride might simply reflect differences in efficacy of SRD5A inhibition, thus correlating with intra-prostatic DHT levels or the calculated androgen index. However, dutasteride was uniformly effective in reducing prostatic DHT, with no DHT levels in treated samples exceeding untreated samples (Supp. 4a). Testosterone levels were more variable, as were measures of the estimated androgen index (Supp. 4b–e). However, plotting samples by tissue DHT, T, or the calculated androgen index did not correlate with segregation into ARG-Lo or ARG-Hi cohorts (Supp. 5). Accordingly, we found no differences in expression of SRD5A1, SRD5A2, or SRD5A3 in ARG-Lo or ARG-Hi groups (Supp. 6), nor any difference in SRD5A2 SNP alleles known to modulate SRD5A2 functional activity (including A49T, P48R, V89L and F194L; data not shown). (20,21) Thus, we could not attribute observed variation in AR-responsive genes to differences in dutasteride effects on DHT reduction.

SRD5A effects on the AR program are highly correlated with cellular AR expression and weakly associated with tissue androgen levels

To further examine prostatic androgen responses to dutasteride, we identified genes in ARG-Hi and ARG-Lo groups most closely associated with sample segregation. With one exception, simply ordering dutasteride-treated samples based solely on AR mRNA abundance partitioned samples into the same ARG-Hi and ARG-Lo categories (Figure 3c) identified by clustering of samples with the 90 androgen-regulated genes (Figure 3a). Using qRT-PCR we confirmed that segregation of dutasteride-treated samples into ARG-Hi and ARG-Lo groups similarly showed relatively high and low expression of AR, PSA and TMPRSS2 (Figure 4a). Moreover, PSA and TMPRSS2 showed a highly significant correlation with AR transcript expression (r^2=0.45 (P<0.001) and r^2=0.52 (P<0.0001), respectively, Figure 4b). Accordingly, known AR-regulated genes predominated among those whose microarray expression profile was most strongly correlated with AR transcript expression (Supp. 7).

In contrast, we observed a much weaker correlation between androgen-regulated genes and tissue androgen levels. Plotting transcript levels against tissue DHT, T, or androgen index yielded modestly significant correlations between TMPRSS2 and DHT (r^2=0.21 P=0.04) and androgen index (r^2=0.28; P=0.01), while correlations for PSA only trended toward significance (P= 0.06 and 0.08, respectively, Supp. 8). In addition, we observed no differences in polyQ and polyG tri-nucleotide repeats (associated with AR trans-activating capacity) in ARG-Lo and ARG-Hi groups (data not shown). (22,23) These observations suggest the primary driver of prostatic androgen response in dutasteride-treated tissues is the level of AR itself.

While AR activity (measured by epithelial expression of androgen-regulated genes) correlated closely with AR transcript levels, it is unknown whether variable AR expression in dutasteride-treated samples reflects steady-state AR status, or if a subset of subjects respond to decreased androgen levels caused by SRD5A inhibition by modulating AR transcript synthesis or stability. As this study did not include pre-and post-treatment samples, we cannot directly address this possibility. However, the wide variation in AR transcript levels in untreated subjects suggests that ‘intrinsic’ AR levels could account for the range of AR expression observed in dutasteride-treated samples. To confirm that AR levels normally exhibit substantial variability, we examined AR transcript abundance in 4 published microarray datasets (13–16) and in microdissected benign epithelium from untreated prostatectomy (n=12) and needle biopsy (n=10) specimens. Consistent with the range of AR expression observed in the present study, AR abundance in untreated prostate samples from published data sets ranged 16-fold (Supp. 9).

As SRD5A inhibition has been associated with histopathologic measures of epithelial cell atrophy in some (but not all) studies (8,24–27), we examined whether differences in AR transcript expression between ARG-Lo and ARG-Hi cohorts might reflect a differential induction of luminal cell atrophy, with relative loss of AR-expressing luminal cells but continued presence of AR-negative basal cells in the ARG-Lo cohort. We found no consistent difference in expression of four basal (TP63, CD44, KRT5 and KRT7) or four luminal cell markers (KRT8, KRT14, KRT17 and KRT18) between ARG-Lo and ARG-Hi cohorts (by qRT-PCR, data not shown), nor did we observe histopathologic differences in relative proportion of epithelial glands demonstrating microcystic atrophy (data not shown).

SRD5A inhibition impacts TMPRSS2 expression in cancerous epithelium

Although androgens and the AR are clearly associated with PCa development and progression, determining specific androgen-regulated genes directly contributing to carcinogenesis has proven elusive. To assess potential functional importance of dutasteride-associated transcriptional changes, we evaluated protein expression of trefoil factor 3 (TFF3, down-

regulated 2.1-fold by dutasteride, Figure 2a) and TMPRSS2, epithelial genes implicated in PCa genesis and/or progression. (17, 18, 28, 29) As previously observed, TFF3 exhibited a spectrum of expression with more intense staining in cancer than benign epithelium. (28) Expression was generally low in control samples and further decreased by dutasteride (Figure 5a,c), with no effect of dutasteride in cancer tissue. TMPRSS2 also stained more intensely in cancer than benign epithelium, with dutasteride reducing staining intensity in both benign and cancer tissues (Figure 5b,c).

The significant, albeit modest, dutasteride-associated reductions in TMPRSS2 transcript and protein levels are noteworthy in view of the frequent chromosomal rearrangements found in PCa and pre-neoplastic lesions placing ETS oncogene family members under control of genes with androgen-regulated promoters such as TMPRSS2 (18). To evaluate potential effects of dutasteride on oncogenic events driven by these rearrangements, we assessed TMPRSS2-ERG fusion status using FISH analysis of triplicate cancer cores per patient. Of samples with sufficient cancer for analysis, we identified theTMPRSS2-ERG fusion in 49% of cancer cores from untreated patients (n=19 evaluable) and in 57% and 25% of the low (n=18 evaluable) and high-dose (n=16 evaluable) dutasteride-treated groups, respectively (Supp 10a; the number of positive and negative cores for each case is given in Supp. 10b). The frequency of fusion-positive cancer cores was significantly lower in high-dose dutasteride vs. untreated samples (p=0.048). The significance of this observation is uncertain given the relatively short duration of dutasteride treatment prior to prostatectomy, but is consistent with the hypothesis that cancers harboring TMPRSS2-ERG fusions may be sensitive to modulation by dutasteride.

DISCUSSION

Prostate cancer prevention remains an attractive approach for reducing morbidity and mortality (30), and a substantial impact might be achieved by decreasing the rate at which relatively indolent foci of malignancy (present in the majority of men with advancing age) progress to clinically detectable disease. (31) Increasingly, there is momentum to ‘treat’ these common low-grade cancers with surveillance plans incorporating administration of natural products or pharmaceuticals with minimal side-effects and low attendant risk profiles. (27,32) In this context, evaluation of SRD5A inhibitors has a strong rationale centered on a mechanism-based role for androgens and the AR in maintaining physiological functions of benign and neoplastic prostate epithelium.

The PCPT formally tested the hypothesis that decreasing SRD5A activity would lower PCa incidence. Compared with placebo, finasteride reduced the 7-year period prevalence of PCa by 25% (33). However, 803 of 4368 finasteride-treated men (~18%) were diagnosed with prostate cancers spanning low and high-grade histologies, as were ~16% of dutasteride-treated men in the REDUCE study. (7) Many unexplained questions remain regarding preventive versus regressive effects of SRD5A inhibition on PCa development and progression. Importantly, it is unknown why SRD5A inhibition was not effective in all individuals nor what factors underlie the variable outcomes observed.

Finasteride and dutasteride substantially reduce intra-prostatic DHT levels and have been shown to reduce prostate volume and improve voiding symptoms related to benign prostatic hypertrophy. (34–38) However, measureable effects of these agents toward prostate epithelium and vasculature are not consistent. Remarkably, studies including analyses of prostate samples from the PCPT (6) have documented that histological assessments are not capable of distinguishing tissues treated with SRD5A inhibitors vs. placebo. A report by the Proscar Long-Term Efficacy and Safety Study group concluded that no significant histological differences were discernable in either benign or cancerous prostate tissues when comparing finasteride and placebo. (24) Randomized studies of dutasteride treatment have not found reproducible

associations with measures of micro-vessel density, epithelial cell atrophy, proliferation rates or apoptosis. (8,25) While these results may partly reflect the substantial normal variability in aging-related androgen decline and attendant tissue effects in untreated individuals, other influences may inhibit or accentuate responses to SRD5A inhibitors, with biological consequences important for the efficacy of this strategy in PCa prevention and treatment.

In this study we identified several possible mechanisms by which dutasteride could modify the development and progression of PCa. Several gene products with known roles or associations with prostate carcinogenesis were altered by dutasteride therapy: prostate epithelium from treated subjects expressed elevated levels of IGFBP3 transcripts (39) and lower levels of TFF3 (28,29) and TMPRSS2. (10,17) Reduced TMPRSS2 expression is particularly relevant in view of recurrent genomic rearrangements involving TMPRSS2 and members of the ETS oncogene family that occur in more than 50% of all prostate cancers. (18) In support of the hypothesis that cancers harboring TMPRSS2-ETS-family rearrangements may be driven by AR signaling, we found the frequency of cancers with TMPRSS2-ERG fusion was reduced in the cohort of patients treated with high-dose dutasteride, although the implication of this observation is limited by the relatively small sample size and short treatment duration.

Importantly, the substantial degree of heterogeneity in the molecular response to dutasteride therapy was unanticipated. Despite near-uniform reduction of tissue DHT levels to the lowest limits of assay detection, many dutasteride-treated tissues continued to exhibit robust activity of the AR-gene expression program. Moreover, we found only weak associations between AR activity and measures of tissue testosterone, DHT, or a composite metric of androgen concentrations. Notably, the molecular feature most closely associated with status of the AR expression program was the level of AR itself.

AR expression levels have previously been found to contribute to development of castration-resistant prostate cancer (CRPC). Using isogenic PCa xenograft models, Chen *et al* found a modest increase in AR was the only change consistently associated with development of resistance to anti-androgen therapy. (40) Elevated AR levels converted cancers from androgen-sensitive to castration-resistant, with cancer growth proceeding by enhanced output of the signal contributed by low residual androgens.

While oncogenic events such as AR gene amplification or dys-regulated kinase signaling may lead to increased AR transcript expression in PCa (41,42), our studies of benign epithelium demonstrates that a wide range of AR expression normally exists (or can be induced) in prostatic epithelium, without a requirement for aberrant genomic or epigenetic events that accompany neoplastic growth. The wild-type AR gene has been shown to function as a self-regulating transcription factor capable of binding to response elements within its coding region leading to increased mRNA levels. (43) Thus, natural variations in levels of co-activator/co-repressors, or polymorphisms involving genomic regulatory sites in the AR itself may contribute to intrinsic differences in AR regulation.

A potential limitation of our study is that we evaluated the molecular impact of SRD5A inhibition on benign epithelium of men with known PCa. However, a study evaluating chemo-prevention in subjects without known cancer is also likely to harbor a certain percentage of men with undiagnosed PCa (as demonstrated by the PCPT). Moreover, emerging data implicate a field cancerization effect underlying multi-focal PCa development (44), suggesting that our findings in benign appearing epithelium of men with cancer are likely to be relevant to the effect of SRD5A inhibition in modulating progression of pre-malignant lesions to overt cancer.

Variation in the molecular program of AR gene regulation has important implications for the optimal use of SRD5A inhibitors in PCa prevention and treatment. Our data suggest the hypothesis that under conditions of relative androgen depletion, high AR levels in benign

prostate epithelium can maintain AR transcriptional network activity, whereas low AR levels cannot compensate for low ligand concentrations. The lack of compensatory ability in some individuals could impact development and/or progression of initiated/pre-neoplastic prostate lesions (possibly those harboring an androgen-driven TMPRSS2-ERG rearrangement). Although mechanisms responsible for variation in AR transcript expression have not been defined, our data lead to the hypothesis that pre-treatment tissue AR levels may predict response to SRD5A-directed therapies, a question that can be tested in an appropriately designed clinical study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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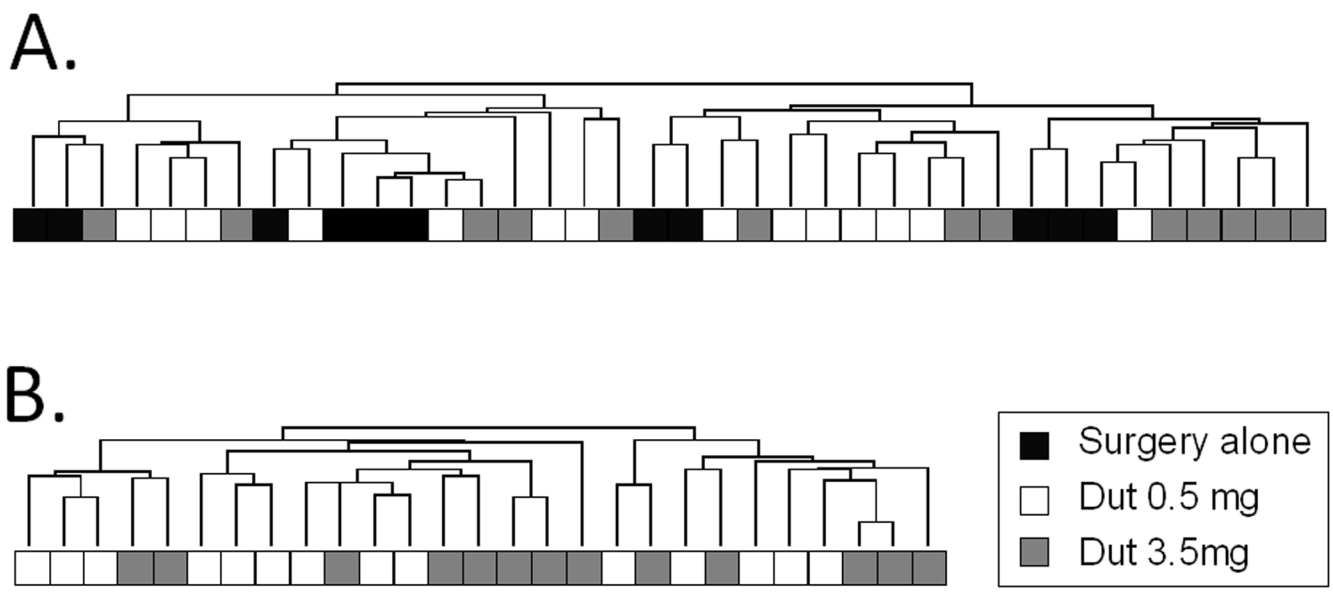

Figure 1. Unsupervised clustering of untreated and dutasteride-treated samples based on prostate epithelial gene expression

Samples from patients undergoing surgery alone (black squares) or treated with 0.5 or 3.5 mg dutasteride (white and gray squares) were ordered based on unsupervised clustering using the 1000 most variably expressed genes. Dendrograms depict relationship of all samples **(a)** or dutasteride-treated samples **(b)**.

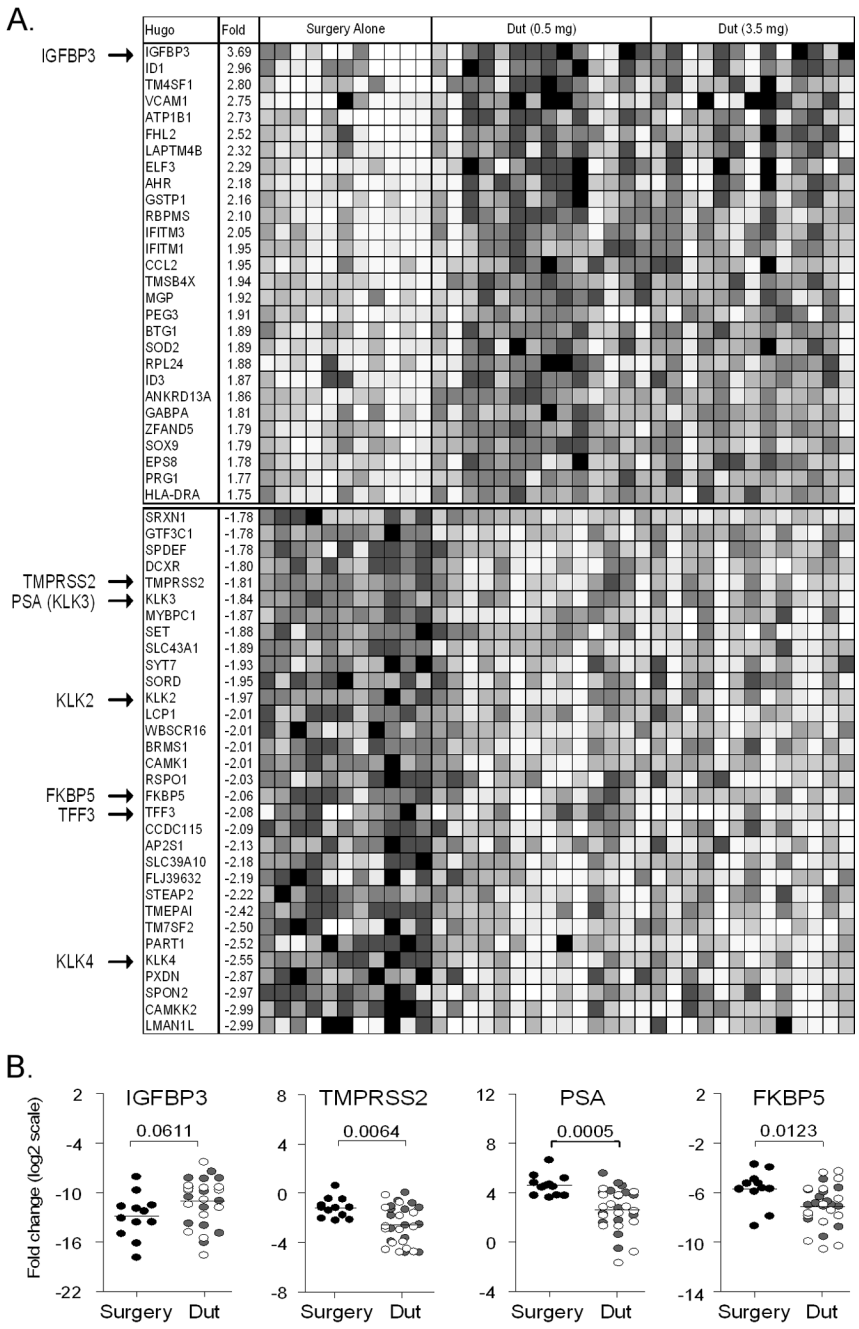

Figure 2. Differential gene expression in microdissected prostate epithelium from untreated and dutasteride-treated patients

(a) The top 60 up or down-regulated genes identified in a supervised two-sample t test between untreated (surgery alone) and combined dutasteride-treated samples (≥1.75 fold, FDR <5%). The full list of 120 genes differentially regulated by ≥1.5 fold is provided in Supp. 3. Grey scale represents genes with lower expression as white, no change as grey and higher expression as black. **(b)** Confirmation of microarray data by qRT-PCR of indicated genes. Y-axis is fold change in expression (relative to RPL13A). Horizontal bars indicate mean values. Two sample t-tests were used to compare difference in mean cycle thresholds between untreated (Surgery)

and dutasteride-treated (Dut) samples with p values as depicted. (Black circles untreated samples, white circles 0.5mg Dut, and grey circles 3.5mg Dut.)

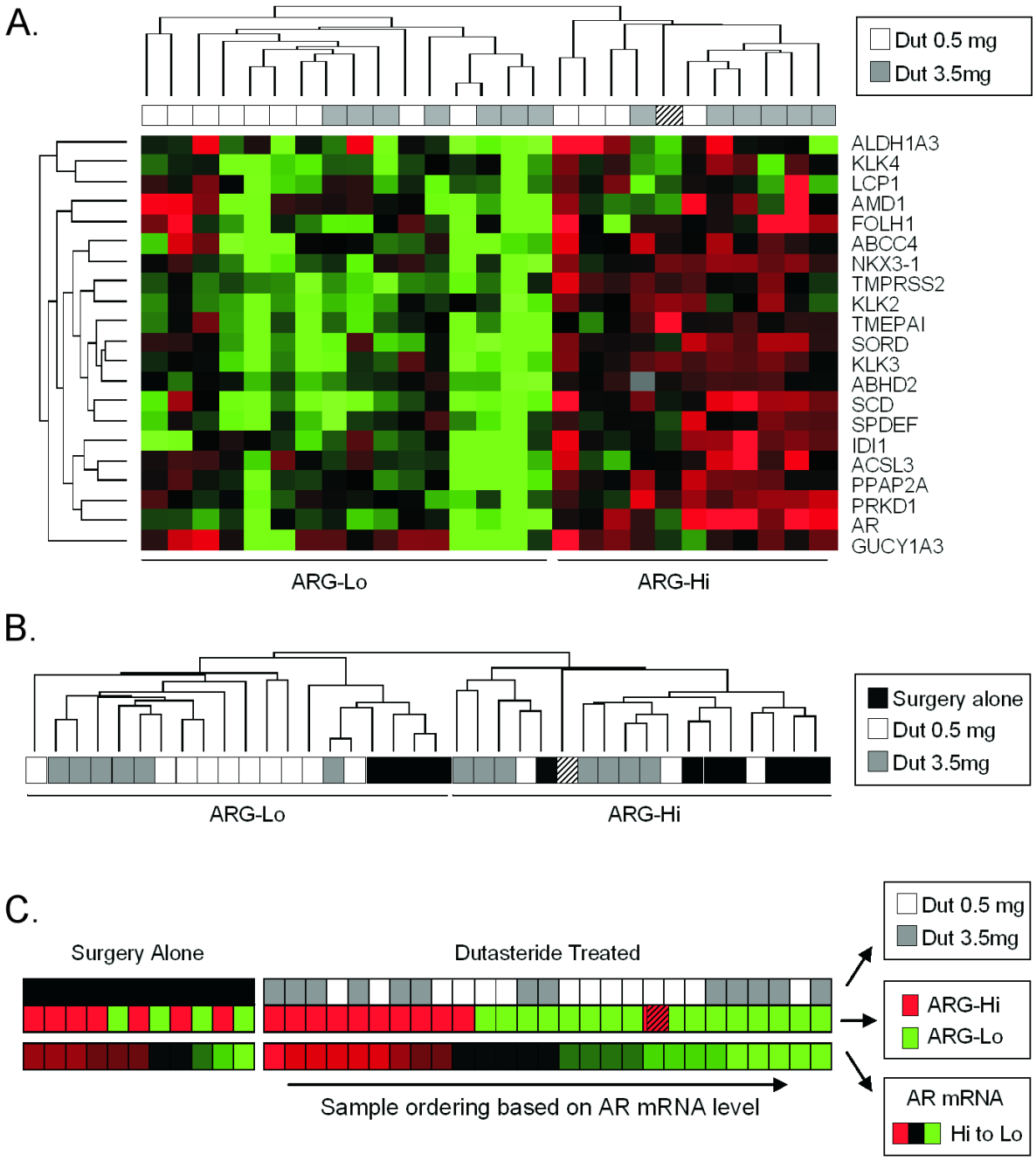

Figure 3. Unsupervised clustering of dutasteride-treated samples based on expression of androgen-regulated genes

Benign prostate epithelial samples from dutasteride patients (white squares 0.5mg; gray squares 3.5 mg) were ordered based on unsupervised hierarchical clustering using 90 androgen-regulated genes. **(a)** The dutasteride-treated samples segregated into two groups based on low expression of androgen-regulated genes (ARG-Lo; green shaded portion of heat map) vs. higher expression of androgen-regulated genes (ARG-Hi; red shaded). Depicted is a node containing a subset of 90 androgen-regulated genes used to perform clustering. **(b)** Unsupervised clustering of entire sample set (including untreated cases, black squares) using the same 90 androgen-regulated genes. **(c)** Surgery Alone and dutasteride-treated samples were

separately ordered based on AR transcript expression level (microarray data, red denotes higher and green lower expression). Dutasteride treatment and whether each sample was designated as high (ARG-Hi) or low (ARG-Lo) for expression of androgen-regulated genes from the cluster analysis in **(a)** is indicated. Crosshatching denotes the one sample with lower AR transcript expression that was assigned to ARG-Hi in the cluster analysis.

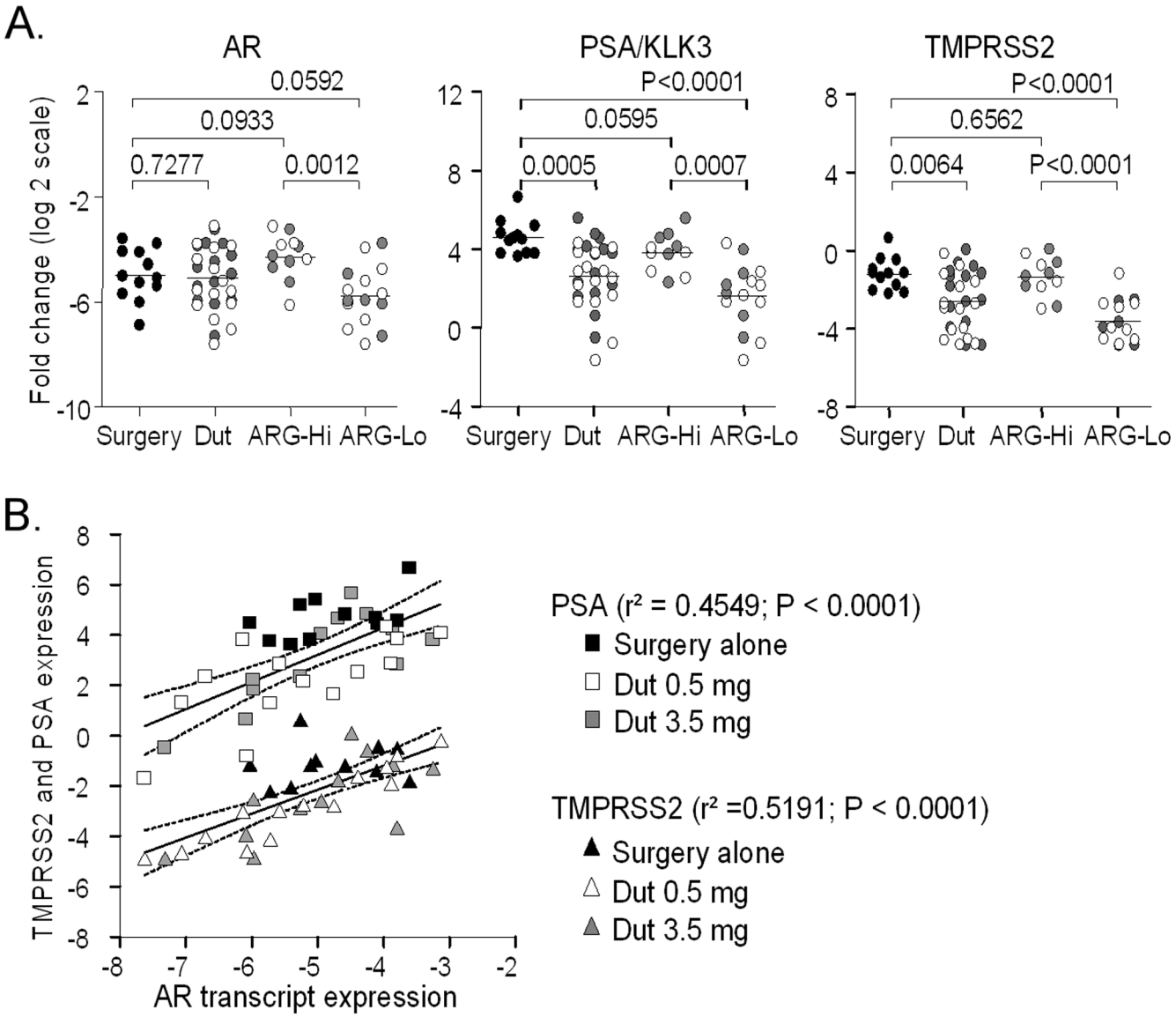

Figure 4. Comparison of androgen responsive gene status with AR transcript abundance

(a) Dot plots showing expression of indicated genes by qRT-PCR in Surgery alone and dutasteride-treated groups; designation of dutasteride-treated samples into ARG-Hi and ARG-Lo is based on clustering of microarray data in Figure 3(a). (Black circles untreated samples; white circles 0.5mg Dut; grey circles 3.5mg Dut.) **(b)** The linear regression of AR transcript expression with that of PSA (squares; slope = 0.4203) and TMPRSS2 (triangles; slope = 0.5442) by qRT-PCR.

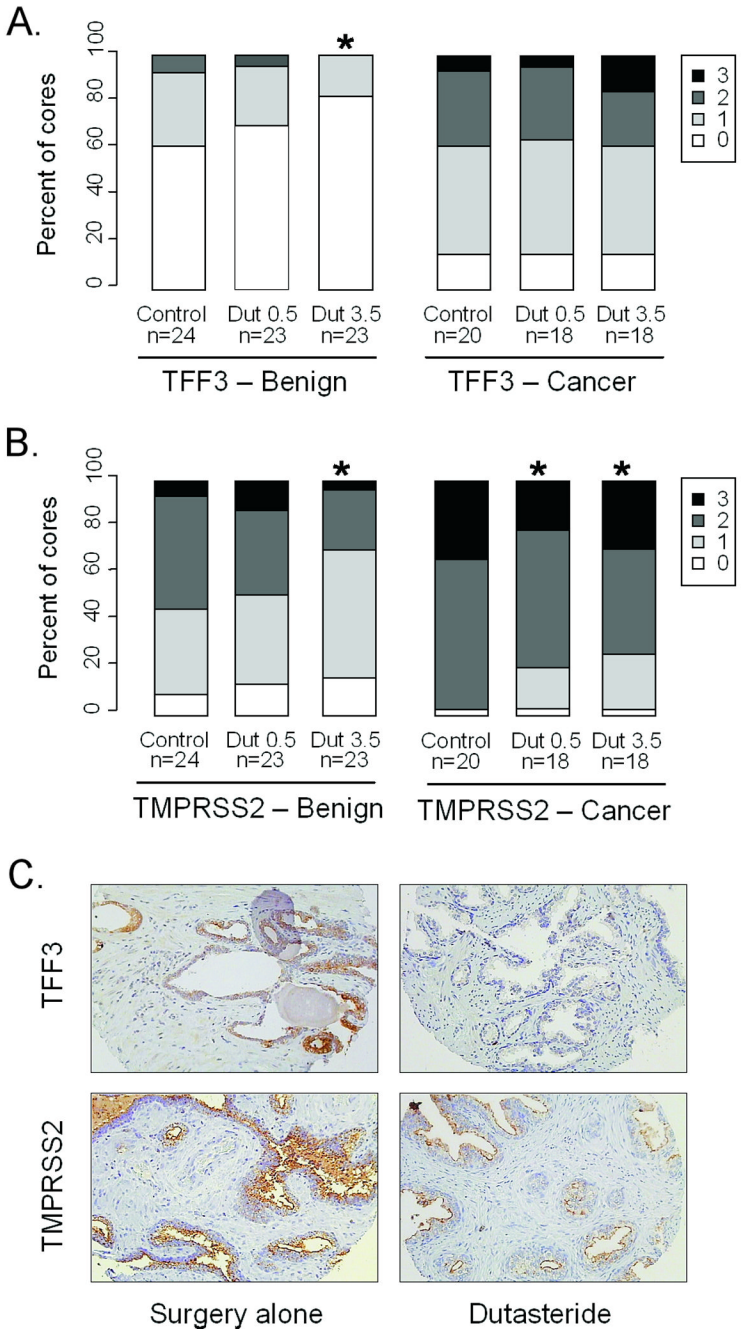

Figure 5. Analysis of TFF3 and TMPRSS2 expression in neoplastic prostate epithelium

TFF3 and TMPRSS2 expression was evaluated using a TMA comprising triplicate benign and cancer cores per patient. Staining was scored on a 4-point scale from none (0) to high (3). The distribution of cores in each treatment group displaying no (0), low (1), moderate (2) or high (3) intensity staining is shown for TFF3 **(a)** and TMPRSS2 **(b)**. Staining intensity in control and treatment groups was compared using a logistic regression model to account for multiple observations per patient, with statistically significant changes indicated (*). Decreases in TFF3 staining were significant in the benign tissue (Dut 3.5 group; p <50.01), and for TMPRSS2 in the benign (Dut 3.5 group; p= 0.03) and cancer tissue (Dut 0.5; p=0.04 and Dut 3.5; p=0.02).

Representative images (20x) showing TFF3 and TMPRSS2 staining in benign untreated (surgery alone) and dutasteride-treated cores **(c)**.

Table 1

Serum and tissue androgen levels after dutasteride treatment

	Surgery alone mean (+/− stdev)			Dutasteride 0.5 mg mean (+/− stdev)			Dutasteride 3.5 mg mean (+/− stdev)		
	Pre	**Post**	**P value***	**Pre**	**Post**	**P value***	**Pre**	**Post**	**P value***
Serum (ng/dl)									
DHT	37 (17)	39 (16)	NS	43 (20)	3.86 (2.1)	<0.001	43 (17)	3.08 (1.6)	<0.001
Testosterone	370 (124)	385 (150)	NS	419 (147)	477 (181)	P=0.026	389 (123)	476 (147)	P=0.006
Tissue (ng/g)									
DHT		3.33 (1.15)			0.23 (0.12)	<0.001		0.04 (0.02)	<0.001
Testosterone		0.07 (0.08)			3.40 (1.26)	<0.001		2.83 (0.89)	<0.001
Tissue androgen index**									
T+2xDHT		6.7			3.9			2.9	
T+5xDHT		16.7			4.6			3.0	
T+10xDHT		33.4			5.7			3.2	

* p value is for comparison of pre vs post treatment measurements for serum, and for comparison of treated vs surgery alone values for tissue.

** Calculation of tissue androgen activity based on estimating DHT to be 2, 5 or 10 fold more potent than testosterone