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Neoadjuvant-intensive androgen deprivation therapy selects for prostate tumor foci with diverse subclonal oncogenic alterations

Adam G. Sowalsky^{1,2,†}, Huihui Ye^{3,†}, Manoj Bhasin⁴, Eliezer M. Van Allen⁵, Massimo Loda⁶, Rosina T. Lis⁶, Laleh Montaser-Kouhsari³, Carla Calagua^{1,3}, Fen Ma¹, Joshua W. Russo¹, Rachel J. Schaefer¹, Olga S. Voznesensky¹, Zhenwei Zhang⁵, Glenn J. Bubley¹, Bruce Montgomery⁷, Elahe A. Mostaghel⁷, Peter S. Nelson^{7,8}, Mary-Ellen Taplin⁶, and Steven P. Balk^{1,*}

¹Department of Medicine, Beth Israel Deaconess Medical Center, Boston, MA 02215

²Laboratory of Genitourinary Cancer Pathogenesis, National Cancer Institute, NIH, Bethesda, MD, 20892

³Department of Pathology, Beth Israel Deaconess Medical Center, Boston, MA 02215

⁴Genomics, Proteomics, Bioinformatics and Systems Biology Center, Beth Israel Deaconess Medical Center, Boston, MA 02215

⁵Lank Center for Genitourinary Oncology, Dana-Farber Cancer Institute, Boston, MA 02215

⁶Center for Molecular Oncologic Pathology, Brigham and Women's Hospital, Boston, MA 02215

⁷Medical Oncology Division, University of Washington, Seattle, WA, 98105

⁸Fred Hutchinson Cancer Research Center, Seattle, WA, 98105

Abstract

Primary prostate cancer (PCa) can have extensive microheterogeneity, but its contribution to the later emergence of metastatic castration-resistant PCa (mCRPC) remains unclear. In this study, we microdissected residual PCa foci in radical prostatectomies from 18 men treated with neoadjuvant intensive androgen deprivation therapy (leuprolide, abiraterone acetate, and prednisone) and analyzed them for resistance mechanisms. Transcriptome profiling showed reduced but persistent androgen receptor (AR) activity in residual tumors with no increase in neuroendocrine differentiation. Proliferation correlated negatively with AR activity but positively with decreased RB1 expression, and whole exome sequencing (WES) further showed enrichment for RB1 genomic loss. In 15 cases where 2 or 3 tumor foci were microdissected, WES confirmed a common clonal origin but identified multiple oncogenic alterations unique to each focus. These findings show that subclones with oncogenic alterations found in mCRPC are present in primary PCa and are selected for by neoadjuvant-intense androgen deprivation therapy. In particular, this study indicates that subclonal RB1 loss may be more common than previously appreciated in intermediate- to high-risk primary PCa and may be an early event, independent of neuroendocrine

*To whom correspondence should be addressed: sbalk@bidmc.harvard.edu.

†Co-first authors

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differentiation, in the development of mCRPC. Comprehensive molecular analyses of primary PCa may detect aggressive subclones and possibly inform adjuvant strategies to prevent recurrence.

Keywords

prostate cancer; androgen receptor; neoadjuvant therapy; castration-resistant prostate cancer; tumor heterogeneity

Introduction

The androgen receptor (AR) plays a central role in prostate cancer (PCa) and most patients with metastatic PCa respond initially to androgen deprivation therapy (ADT), but they invariably relapse despite castrate systemic androgen levels (castration-resistant prostate cancer, CRPC) with tumors that express high levels of AR and AR regulated genes. Increased intratumoral androgen synthesis is a major mechanism driving AR in CRPC (1–5), and responses can be obtained by further suppression of androgen synthesis using agents such as abiraterone that block CYP17A1 or by more effective AR antagonists such as enzalutamide. Based on the hypothesis that early use of ADT may improve outcomes, previous clinical trials have examined ADT with a GnRH agonist prior to radical prostatectomy (RP) in men with clinically localized disease. Although this neoadjuvant GnRH agonist therapy was generally found to reduce tumor volume, complete pathological responses were rare and there was no clear improvement in survival (6). Significantly, while treatment with a GnRH agonist decreases serum androgens, substantial levels persist in the prostate due to increased conversion of adrenal derived DHEA to testosterone and DHT by normal prostate as well as by tumor cells (7,8).

Based on these observations we hypothesized that efficacy in previous neoadjuvant ADT trials may have been compromised due to inadequate suppression of intraprostatic androgens, and that responses may be improved by more robust suppression of androgen synthesis. To test this hypothesis we conducted a phase 2 trial of neoadjuvant leuprolide for 24 weeks in combination with abiraterone acetate and prednisone (referred to subsequently as leuprolide plus abiraterone) for 12 or 24 weeks prior to RP. As reported recently, we confirmed that the addition of abiraterone further markedly reduced intraprostatic androgen levels and appeared to improve responses relative to historical controls using single agent GnRH agonists (9). Nonetheless, residual PCa was found in the majority of patients, with only a small number of patients demonstrating complete pathological responses. Moreover, substantial nuclear and cytoplasmic AR expression was detected by immunohistochemistry in most cases, suggesting that AR activity still may persist and contribute to residual disease. In this study we have exploited RP specimens containing residual tumor from this trial in order to identify possible mechanisms of resistance to the combination leuprolide-abiraterone therapy.

Materials and Methods

Tissue procurement

Radical prostatectomy (RP) tissue of trial cases was acquired as part of our clinical trial (NCT00924469, DF-HCC study 09-107). The studies were conducted in accordance with recognized ethical guidelines (U.S. Common Rule), were approved by the DF/HCC IRB, and written informed consent from the patients was obtained. For matched untreated controls, tissue from RPs performed between 2010 and 2016 at Beth Israel Deaconess Medical Center (BIDMC) under standard-of-care were collected and de-identified in accordance with BIDMC IRB protocol #2010-P-000254. All trial patients received 24 weeks of leuprolide prior to RP, and those patients received either 12 weeks or 24 weeks of simultaneous treatment with abiraterone acetate plus prednisone leading up to surgery. Control cases matched the trial cases on pre-RP characteristics including ethnicity, serum PSA level, biopsy tumor grade, number of positive cores, and maximum cancer percentage on positive cores. Clinicopathologic characteristics of the two cohorts are listed in Table 1. Tumor was graded using both new Grade Group (GG) system and Gleason Score (GS) as recommended by the 2014 International Urological Pathology (ISUP) consensus (10). Prostate tissue was fixed in formalin, processed, and embedded in paraffin using standard methods. For each case, one tissue block with the largest dimensions of the index tumor was selected for immunohistochemistry (IHC) and in situ hybridization.

Immunohistochemistry evaluation

IHC stains were scored semiquantitatively as follows. Ki-67 IHC score was defined as the highest Ki-67 percentage score per high power field across the tumor sections. ERG was scored as 0 (negative) and 1 (positive). PTEN loss scores were derived as follows: 1) PTEN binary score was scored as 0 (retained, or reduced staining, or negative staining in <5% tumor cells), and 1 (negative staining in ≥5% tumor cells); 2) percentage score was assigned based on percentage of tumor cells with negative staining as: 0 (no negative staining), 1 (1–9%), 2 (10–49%), and 3 (≥50%); 3) Final PTEN loss IHC score (0–3) was derived by the binary score multiplied by the percentage score. pAkt Ser473 was scored as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong), based on the most predominant intensity pattern. Synaptophysin and chromogranin was scored as 0 (negative), 1 (<1–4%), 2 (5–24%), and 3 (25–49%), and 4 (≥50%). For AR, AR-V7, PSA, and NKX3.1, IHC scores were derived as follows: 1) immunointensity score was assigned as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong), based on the most predominant intensity pattern; 2) percentage score was assigned based on percentage of tumor cells demonstrating the most predominant intensity pattern as: 0 (negative), 1 (1–9%), 2 (10–49%), and 3 (≥50%); 3) final IHC score (0–9) was derived by the immunointensity score multiplied by the percentage score. All immunostains were evaluated by a board-certified pathologist (HY) and a trained physician investigator (LM or CC).

Statistical Analysis

Stata 12.1 (StataCorp, College Station, TX) was used for statistical analysis. Characteristics between groups were compared using Pearson chi-square and Fisher's exact tests for

categorical variables, and Wilcoxon rank-sum test for nonparametric continuous and ordinal variables. A $P < 0.05$ was considered statistically significant.

Transcriptome analyses

Samples were stained with H&E and PIN-4, and serial sections were stained with Paradise Plus stain and laser capture microdissected (LCM) as previously described (11). Regions of residual or untreated tumor (or accompanying regions not involved with tumor) were identified by a board-certified pathologist (HY or RL) and images of capture tissue were verified prior to extraction. RNA and DNA were extracted simultaneously using the AllPrep DNA/RNA FFPE Kit (Qiagen) with modifications as described previously (12). 50ng of total RNA from LCM tissue was amplified and prepared into sense-strand cDNA as previously described (12). Labeled libraries were hybridized onto Affymetrix Human Transcriptome Array 2.0 GeneChips. In addition to foci of residual tumor, three foci of tumor from untreated cases were microdissected as control cases, and used in conjunction with microarray data from 26 foci of similarly-processed samples (12) for a total of 29 control samples.

Genomic Analyses

1–100ng of genomic DNA from LCM tissue was prepared into libraries using the HyperPrep kit (KAPA) modified to include reagents from the Agilent SureSelectXT2 kit, to enable low-input amounts of FFPE-derived DNA. Following sonication (Covaris), clean-up and end-repair, partial SureSelect Agilent linkers were ligated to the DNA at a molar ratio of 10:1. PCR was performed until 1 µg of pre-capture library was generated. Hybridization to the SureSelect Exome V5 was performed in solution for 48 hours, and additional amplification was performed to generate at least 10 µl of a 10nM library. Libraries were quantified with the KAPA Illumina QC kit, pooled in equimolar ratios, and sequenced on an Illumina HiSeq2500. Alignment, deduplication, local realignment, and quality score recalibration were performed with BWA, PICARD and GATK. Mutation calling and annotation was performed using muTect2 and Oncotator (13,14), and mutations were confirmed by VarScan (15).

Accession Numbers

Sequencing and microarray raw data were deposited into dbGaP, accession phs001399.v1.p1. Gene expression data were deposited into GEO, accession GSE102124. Methods for machine-aided IHC quantification, criteria for calling genomic alterations, and criteria for calling differentially expressed genes and pathways are provided in Supplementary Data.

Results

Proliferation and AR Signaling are Reduced in Residual Tumors after Neoadjuvant Leuprolide and Abiraterone

As our study (NCT00924469) recently reported (9), while neoadjuvant leuprolide plus abiraterone substantially reduced tumor volumes, 52 (93%) of 56 trial patients still had foci of residual tumor detected in their RP specimens. Among these 52 patients, residual tumors

had a median cross-sectional greatest dimension of 1.5 cm (IQR: 1.0 – 2.5 cm), and median residual cancer burden (RCB) of 0.17 cm³ (IQR: 0.02 – 0.86 cm³). Residual tumors measuring > 0.5 cm in the greatest dimension were found in 48 (86%) of patients. In most cases the residual tumors were pathologically acinar adenocarcinomas, not otherwise specified (NOS) (Figure S1A), except two cases that contained a component of adenocarcinoma with Paneth cell-like neuroendocrine differentiation (Supplementary Figure S1B). These two cases had tumor sizes of 2.5 cm and 1.5 cm in the greatest dimension and had RCB of 1.48 and 0.19 cm³, respectively. The tumor components with Paneth cell-like neuroendocrine differentiation expressed neuroendocrine markers including synaptophysin and chromogranin, with reduced expression of AR and AR targets (NKX3.1 and PSA) in comparison with the co-existing component of adenocarcinoma, NOS (Supplementary Figure S1B).

For the 49 trial cases that had available tissue for further immunohistochemistry (IHC) characterization, we examined IHC profiles of residual tumors to assess cell proliferation and AR activity. Adequate tissue from baseline core biopsies was not available in most cases, so trial cases were compared with 49 untreated control cases with matched baseline clinicopathologic characteristics (Supplementary Table S1). Cell proliferation rate, measured by the Ki-67 index on IHC (Figure 1A), was significantly reduced ($P < 0.0001$, Wilcoxon rank sum test) in treated tumors compared to untreated controls (Figure 1B). Amongst the treated cases, the 4 cases with the highest Ki-67 indices were in the 24-week abiraterone arm (cases 513, 601, 609, and 006, Figure 1C). In some cases, spatially separated tumor foci of the same index tumor demonstrated markedly diverse Ki-67 proliferation indices (such as case 513, depicted in Figure 1A). AR expression with variable nuclear and cytoplasmic levels was readily detected in 48 (98%) of residual tumors (Figure 1D; **see also** Figure S1B). However, nuclear AR was significantly reduced compared to untreated controls, consistent with effects of the intense androgen deprivation therapy ($P = 0.0175$, Wilcoxon rank sum test). Nuclear AR IHC scores in the 24-week abiraterone arm showed a trend toward reduction compared to that of the 12-week arm (Figure 1D).

Recent studies indicate that the emergence of CRPC and resistance to abiraterone and enzalutamide is associated with increased expression of AR splice variants that delete the ligand binding domain, with AR-V7 being the most commonly expressed variant (16). *In situ* hybridization (ISH) with probes against the AR-V7 mRNA showed focal increases in AR-V7 expression, which were concordant with increases in total AR mRNA as indicated by a probe recognizing a region (nucleotides 1786–2998) shared by the full length AR and AR variants (Figure 1E). Immunostaining with an AR-V7 antibody similarly demonstrated focal areas of high AR-V7 expression in the treated tumors (Figure 1F). However, focal staining with the AR-V7 antibody also was observed in the control untreated tumors, and while there was a trend towards increased AR-V7 by IHC in the treated versus control untreated tumors, the increase was not significant (Figure 1G).

Expression of the tightly AR regulated PSA (KLK3) protein was significantly reduced in the treated tumors, suggesting decreased AR activity ($P = 0.0302$, Wilcoxon rank sum test) (Figure 1H). In contrast, expression of the AR regulated protein NKX3.1 was upregulated in treated cases ($P < 0.0001$, Wilcoxon rank sum test) (Figure 1I). ERG immunostaining to

detect expression of the AR-regulated *TMPRSS2:ERG* fusion gene also was positive in comparable numbers of treated versus control tumors (Supplementary Table S1), although treatment was associated with reduced ERG intensity in treated fusion-positive tumors (consistent with lower AR activity). Overall these findings indicated that reduced, but still substantial AR activity persisted in the residual tumor foci.

Immunohistochemistry shows heterogeneous alterations in oncogenic pathways

We reported previously that phosphorylation of ErbB3 was increased in a subset of these residual tumor foci, suggesting that increased ErbB2 activity was a mechanism contributing to resistance (17). To explore further oncogenic mechanisms of resistance, we carried out IHC for additional proteins or pathways known for their enrichment in metastatic CRPC (18). PTEN loss was observed in 47% of residual tumors, as defined by negative PTEN staining in >5% of tumor cells (Figure 2A, Table 1). In 20% of residual tumors this PTEN loss was identified in 5–50% of tumor cells, indicating PTEN loss was subclonal in those cases. Not surprisingly, PTEN loss co-occurred with increased phosphorylation of Akt (Ser473), an indicator of PI 3-kinase pathway activation (Figures 2A–C). PTEN loss also was positively associated with Ki-67 proliferation index (Figure 2D). Compared to untreated tumors, however, treated tumors did not show a significant increase in PTEN loss, either by proportion of cases with PTEN loss or by proportion of tumor cells with PTEN loss (see Table 1). Tumor foci with pRb loss were observed in 3 of 44 (7%) residual tumors examined, with all three cases demonstrating focal/subclonal pRb loss (Figure 2E). Of these three cases (cases 505, 513, and 654), one had an outlier high Ki-67 proliferation index on IHC (case 513, Ki-67 index 50%, see Figure 1B,C).

As long-term ADT promotes neuroendocrine differentiation (NED) in metastatic CRPC, we also examined the influence of the neoadjuvant ADT on NED in tumors that were morphologically acinar adenocarcinomas (as noted above, two cases contained a component with Paneth cell-like neuroendocrine differentiation that were synaptophysin positive, see Supplementary Figure S1B). Synaptophysin expression in scattered individual cells or foci-specific groups of cells was present in treated tumors, but also in matched untreated controls (Supplementary Figure S1C). There was no significant difference in synaptophysin or chromogranin IHC scores between treated and untreated tumors (Pearson chi-square test). Further, we compared the proportions of synaptophysin-positive cells in treated and untreated tumors in all cases that demonstrated ≥5% synaptophysin immunoreactivity by performing computational quantitative analysis on digitalized synaptophysin-stained slides. Consistent with a treatment effect, the results showed more synaptophysin positive cells in untreated cases, but there was no significant enrichment in treated residual tumors compared to untreated controls as a proportion of tumor cells present (Supplementary Figure S1D and S1E).

Taken as a group, these studies indicate that residual tumors had significantly lower Ki-67 and reduced expression of nuclear AR and AR target genes, but no significant increases in AR-V7 expression, PTEN loss, or markers of NED. However, there was substantial intratumoral heterogeneity in residual tumors, as indicated by distinct Ki-67 proliferation indices among foci, focal increases in AR-V7, subclonal losses of PTEN, focal losses of

pRb, and foci-specific NED in subsets of cases. These findings indicated that spatially separated tumor foci may harbor different drivers of resistance.

Substantial AR Transcriptional Activity Persists in Residual Tumor Foci but is not Driving Proliferation

Together the above results suggested that diverse mechanisms may be contributing to resistance in individual tumors, especially in cases where there were morphologically or immunohistochemically distinct residual tumor foci. To further address mechanisms of resistance, in a series of trial cases we used laser capture microdissection (LCM) to purify residual tumor in one to three discrete tumor foci from the formalin fixed and paraffin embedded (FFPE) RP specimens, in conjunction with a distant area that did not contain tumor. Clinicopathologic characteristics of the samples that were microdissected are reported in Supplementary Table S1.

Both RNA and DNA were then extracted from each microdissected tumor focus and corresponding uninvolved benign focus for transcriptome analysis and whole exome sequencing (WES, see below). In cases where adequate RNA was obtained from the microdissected tumor foci, global gene expression was examined on GeneChip Human Transcriptome 2.0 arrays (Affymetrix) and compared with RNA extracted and analyzed previously from similarly microdissected Gleason score 7–9 (with co-existing Gleason pattern 3 and cribriform Gleason pattern 4) control untreated tumors (12). Unsupervised hierarchical clustering based on the 1000 genes with the most variance separated the treated from the control untreated tumor foci (Figure 3A). Interestingly, in some cases tumor foci from the same case did not cluster together, consistent with heterogeneity noted by IHC above.

We next used the gene expression data to assess for proliferation and AR signaling in the residual tumor foci. The proliferation rate, based on a 131-gene index (19), indicated that proliferation was decreased in the residual tumors, consistent with the Ki-67 IHC data (Figure 3B). Also consistent with the Ki-67 IHC, this proliferation index was significantly lower in the cases treated for 12 weeks versus 24 weeks with abiraterone (Figure 3C). Three of the 5 foci that were highest for the 131-gene proliferation single sample gene set enrichment analysis (ssGSEA) score (513.D16, 513.D15, and 006.C21) were from cases with outlier high Ki-67 indices. Two of the four foci with the highest proliferation rate based on ssGSEA had outlier low levels of *RB1* expression based on the transcriptome data (505.C20, 507.C16), suggesting that decreased *RB1* expression may be a driver of increased proliferation in these residual tumors. Indeed, there was a significant negative correlation ($r = -0.56$) between *RB1* mRNA levels and proliferation index, suggesting that *RB1* downregulation was a major factor driving proliferation (Figure 3D).

To assess AR activity we applied ssGSEA with a panel of 267 androgen regulated genes (20). AR activity overall was lower in the residual tumor foci versus control untreated tumors (Figure 3E), consistent with the IHC results. However, it was still substantial, being equivalent to levels in metastatic CRPC and greater than in a panel of neuroendocrine-like prostate cancers (Figure 3F). AR activity was lower after 24 weeks versus 12 weeks of abiraterone (Figure 3G). Surprisingly, we observed a negative correlation ($r = -0.54$)

between the ssGSEA scores for AR activity and proliferation (Figure 3H). To determine whether this was unique to the neoadjuvant setting, we similarly examined other data sets of primary PCa and metastatic CRPC. In the prostate TCGA and MSKCC 2010 primary PCa datasets there were no significant correlations between the AR and proliferation signatures (Supplementary Figure S2A and S2B) (21,22). Similarly, in the Stanbrough and Stand Up to Cancer metastatic CRPC data sets there were no statistically-significant correlations (Supplementary Figure S2C and S2D) (1,18), while there was a negative correlation (as noted previously) in the FHCRC dataset (Supplementary Figure S2E) (23).

Together these observations indicated that while a basal level of AR activity may be necessary, further increases do not drive proliferation in the neoadjuvant or metastatic setting. Consistent with this conclusion, and with decreased *RB1* expression being a driver of proliferation, there was a positive correlation ($r = 0.67$) between the AR activity ssGSEA score and *RB1* expression (Figure 3I). We further found that decreased *RB1* expression was associated with increased expression of genes that are negatively regulated by pRb, based on an 80-gene *RB1* loss signature derived from CRPC, although the correlation did not reach statistical significance (Figure 3J) (24). Finally, although *RB1* loss has been associated with NED in metastatic CRPC (25), we did not observe any substantial negative correlation between expression of *RB1* and *CHGA* ($r = -0.12$), *CHGB* ($r = -0.12$), or *SYP* ($r = -0.17$). Moreover, *RB1* expression was not decreased in the two tumor foci microdissected from a case with Paneth cell-like neuroendocrine differentiation (case 004, $\log_2$ array intensity of *RB1* mRNA = 7.77 and 7.95).

Altered Transcriptional Programs in Residual Tumors

We next broadly examined gene expression in the residual tumor foci versus control untreated tumors. As shown in the Volcano plot, only a relatively small set of genes were significantly overexpressed in the residual tumor foci (Figure 4A). Genes that were increased by at least 2-fold are shown in Supplementary Table S2. Amongst these, the lncRNA SCHLAP1 has been implicated in PCa and is associated with more aggressive disease (26). Alterations in AR coactivators, corepressors, or other modulators also may be contributing to reconstitution of AR activity in some cases. However, none of the genes in Supplementary Table S2 are established AR coactivators. We also examined expression of a series of genes implicated previously as AR coregulators in CRPC (27), and did not identify any that were significantly increased in the treated versus control tumors (Supplementary Table S3).

Interestingly, although *AR* mRNA is markedly increased in metastatic CRPC with frequent *AR* gene amplification (1,28), it was not consistently increased in the residual tumors. However, there was a positive correlation ($r = 0.55$) between *AR* mRNA and AR activity ssGSEA score (which itself does not include *AR* expression), indicating that AR level was contributing to residual AR activity in treated cases (Supplementary Figure S3A). Genes involved in androgen synthesis, and particularly *AKR1C3*, also are increased in metastatic CRPC (1,2). However, expression of *AKR1C3* had no association with AR activity (Supplementary Figure S3B) and expression of *CYP17A1* (encoding the enzyme targeted by abiraterone) was negatively correlated with AR activity (Supplementary Figure S3C).

Interestingly, *NR3C1* (glucocorticoid receptor) expression, which may drive a subset of AR regulated genes in advanced CRPC (29), was positively correlated ($r = 0.52$) with AR activity (Supplementary Figure S3D). Nuclear GR expression by IHC also was generally significantly increased in the treated tumors compared to untreated controls, ($P = 0.0216$, Wilcoxon rank sum test), with no difference between the 24-week and 12-week arms (Supplementary Figure S3E). Consistent with increased GR protein expression, a comprehensive 121-gene GR activity signature, which has considerable overlap with AR target genes (29), was increased in the treated versus untreated cases, although the difference was not statistically significant ($P = 0.0709$, Supplementary Figure S3F). Moreover, when only the 67 genes that did not overlap with AR activity were retested, the treated cases had significantly greater enrichment for GR activity ($P < 0.0001$, Supplementary Figure S3G). Together these findings indicate that GR activity is generally increased in the treated tumors, particularly towards GR specific genes, and also contributes to the expression of AR regulated genes.

In contrast to the relatively small number of genes that were increased in the residual tumors, a much larger set of genes were expressed at higher levels in the control tumors, with ~300 genes being at least 2-fold higher in the control versus treated cases (Supplementary Table S4). As expected, many of these are AR regulated genes. Pathway analysis indicated that the most significant alterations in the residual tumors were decreases in genes involved in the endoplasmic reticulum stress response, the unfolded protein responses, and lipid biosynthesis (Figure 4B). This finding is consistent with decreased AR transcriptional activity, as a major function of AR is to drive protein and lipid synthesis (30–32). Assessment for upstream regulators of these gene expression changes confirmed a central role for decreased AR activity (Figure 4C). It also indicated that there was decreased activity of XBP1 (a positive regulator of the ER unfolded protein response) (33,34) and NFE2L2 (activated in response to oxidative stress) (35), consistent with decreased endoplasmic reticulum stress (Figure 4C). Interestingly, although XBP1 activity primarily is regulated at the level of splicing, XBP1 mRNA levels were positively correlated with AR activity, consistent with AR activity being a major regulator of these metabolic alterations and possibly indirectly increasing XBP1 by increasing metabolic stress (Figure 4D). Alternatively, the *XBPI* gene may be directly regulated by AR in CRPC (36).

Whole Exome Sequencing Confirms that Foci of Residual Tumor are Clonally Related

As noted above, DNA from microdissected tumor foci was in parallel examined by WES. Somatic copy number alterations (SCNAs) were identified based on segmented WES read depth (Supplementary Figure 4A–O). Consistent with a common clonal origin, many SCNAs were shared between two foci from the same RP specimen (Figure 5A, Supplementary Table S5). This also held true for case 518, in which we microdissected tumor and sequenced DNA from three foci. However, many others were unique to one focus, indicating they were likely subclonal in the initial tumor prior to therapy but were clonal in the individual tumor focus.

We next used somatic copy number, allele frequency, and estimated LCM purity to infer which mutations and small indels were likely to be clonal in each focus, and to identify

those that were shared versus unique in foci from the same RP specimen. In case 518 where we dissected three foci, as well as the cases in which two tumor foci were isolated, we identified multiple shared somatic mutations that we presume to be truncal or present on a large branch of the tumor, confirming a common clonal origin (see Supplementary Table S6). Significantly, in those cases with 2 or 3 microdissected foci, we also identified many somatic mutations or small indels that were present in high frequency in one focus, but were not identified in the other focus (Supplementary Table S7). As with the SCNAs, these were presumably subclonal in the untreated tumor, and reflect focal expansion of the corresponding subclones. Finally, in three cases we microdissected and analyzed only a single focus of tumor and identified high-confidence mutations and indels with potential driver status, which may have been truncal or subclonal in the untreated tumor (see Supplementary Table S8).

Subclonal Driver Alterations and Enriched *RB1* Genomic Loss in Residual Tumor Foci

We had reported previously that increased progesterone in men with metastatic CRPC treated with abiraterone could select for the T878A mutant AR that can be strongly activated by progesterone, and identified the T878A mutation in one of two foci from the initial patient analyzed from this trial (37). Amongst the 18 cases examined here, we found the T878A mutation in only one additional patient (case 006, wherein only one focus was analyzed). We also found an *AR* gain in one of two foci from case 506 (focus C11). This apparent alteration rate for *AR* of ~10% is far less than the reported rate of ~60% in mCRPC (18), possibly reflecting less of a growth advantage for tumor cells with these *AR* alterations with upfront use of aggressive ADT. Alternatively, as there would presumably be no selective advantage for cells with *AR* gene amplification or mutations prior to ADT, their precursor frequency would be very low and it may take a more prolonged period of ADT for these cells to emerge and become dominant clones.

To identify further potential driver alterations to tumor suppressor genes or oncogenes, we next filtered genomic events to large chromosomal changes and a refined list of 727 cancer-related genes. The results are summarized for six cases in Figure 5B, and the remaining 9 cases for which 2 foci were analyzed are depicted in Supplementary Figure S5. For each Euler diagram, the size of the circles for each focus reflects the total number of somatic mutations, small indels and copy number alterations that were identified in each focus, as shown in Supplementary Table S9. Overall, genomic alterations as analyzed in these residual tumor foci presented a pattern of sequential tumor suppressor losses, consistent with spatially distinct foci emerging from subclones with unique oncogenic alterations that were cooperating with truncal events acquired earlier in a common precursor.

Foci from several cases showed discordance for *PTEN* loss. Three cases (505, 512, 518) had deletions of one *PTEN* allele in only one focus, and one case (520) had independent single copy *PTEN* losses (Figure 5B and Supplementary Figure S5). Case 003 had a shared deletion of one *PTEN* allele, which likely emerged earlier during tumorigenesis, and only focus C12f2 from this case lost the second *PTEN* allele (Supplementary Figure S5). In addition, the C12f2 focus of case 003 also harbored single copy losses of *TP53*, *CHD1*, and *BRCA1* that were not detected in 003 focus C12f1. In case 516, one focus (C7) exhibited

homozygous losses of *PTEN* and *ZBTB16* (PLZF), while both genes were intact in the other focus (C12) (Figure 5B). It should be noted that while both foci from this case shared alterations a chromosome 8p loss and 8q gain (encompassing *NKX3.1* and *MYC*, respectively), we did not identify any shared mutations or additional shared SCNAs between these foci, indicating that they may reflect independent tumors. Case 513 had biallelic inactivation of *PTEN* in both foci examined, consisting of a single copy loss and a splice site mutation on the remaining copy (Figure 5B). Case 513 also harbored a homozygous deletion of *RB1* in both foci. Consistent with this being a truncal loss, case 513 had the highest level of proliferation based on Ki-67 expression and the greatest residual tumor volume.

In addition to case 513, *RB1* losses were found in 6 other cases (004, 006, 501, 505, 508, 518), and these losses were discordant amongst foci in 3 cases (505, 508, and 518). Case 508 had a single copy *RB1* loss only in focus C6. This focus also had a homozygous deletion of *CHD1* as well as additional alterations that were not present in the other focus, and had a much greater overall mutational burden than focus C5 (Figure 5B). Case 518 demonstrated a truncal loss of one copy of *RB1* detected in all three foci, but only focus C12 harbored a deletion of the second copy of *RB1* (Figure 5B). All three foci shared gains to chromosome 3q encompassing *PIK3CA* and *PIK3CB*. Interestingly, foci C8C9 and C10 shared a frameshift deletion to *ATM* and a copy-neutral loss-of-heterozygosity (LOH) event to chromosome 11 resulting in biallelic inactivation of *ATM*. Focus C8C9 also harbored a high-level amplification of *HSP90AB1*, which was not observed in other foci.

In case 505, the foci shared a loss of chromosome 13q (encompassing *RB1* and *BRCA2*), with only focus C20 harboring an additional loss on the other 13q resulting in homozygous loss of *RB1* and *BRCA2* (Figure 5B). Interestingly, biallelic loss of *TP53* was observed as a truncal event in this case, as both the C17 and C20 foci harbored single copy losses of chromosome 17p with a shared p.W53* nonsense mutation on the remaining copy. Significantly, although focus C20 was *RB1/TP53* null, there was no evidence of neuroendocrine differentiation based on morphology or transcriptome profiling. Conversely, in case 004 where both foci showed neuroendocrine differentiation, there was only a shared single copy loss of *RB1* and no detectable alteration in *TP53* (Figure 5B).

Overall we observed homozygous loss of *PTEN* that appeared to be truncal in one case (513), and homozygous losses in one focus from cases 003 and 516. For *TP53* we observed truncal homozygous loss in one case (505), a truncal single copy loss in case 513, and single copy loss in one focus from case 003. These frequencies for homozygous inactivation of *PTEN* and *TP53* are very similar to the frequencies reported in the primary PCa TCGA dataset (~17% and 6%, respectively) (21), but less than the frequency reported in the PCa SU2C dataset (~38% and 31%, respectively) (18). In contrast, while *RB1* loss appears to be uncommon in primary PCa (< ~1% in TCGA) (21), we found deep deletions to *RB1* in four foci from three cases (505, 513, 518), and single copy *RB1* losses in 9 foci. This enrichment for biallelic inactivation of *RB1* in these cases versus untreated primary PCa was statistically significant (Figure 6A), and was associated with lower expression of *RB1* mRNA (Figure 6B). This finding, and the negative correlation between *RB1* mRNA and proliferation (see Figure 3D), suggest that the intensive androgen deprivation therapy is selecting for tumor foci that have decreased expression of *RB1* mRNA through genetic or epigenetic

mechanisms as a major mechanism for bypassing the G1/S block in response to androgen deprivation.

In addition to these cases with deep deletions of tumor suppressor genes, we found shallow deletions (indicative of single copy losses) in multiple other foci. For example, case 004 (Paneth cell-like morphology) had shared shallow deletions of *APC*, *CHD1*, *RAD50*, *RB1*, and *CDKN1B*, while one focus from this case had additional single copy losses of chromosome 8p, *MSH2*, and *MSH6* (which were associated with an increased mutational burden) (see Figure 5B). Although the significance of these single copy losses is less clear, many of these tumor suppressors demonstrate haploinsufficiency, and their loss also may cooperate with other epigenetic mechanisms or complimentary alterations in other genes (such as losses to *RB1* and *CDKN1B* in case 004) to contribute to tumor survival and growth. Overall these data indicate that the intense androgen deprivation therapy selected for discrete foci of tumor containing genomic alterations that contribute to resistance, which may contribute to the eventual emergence of metastatic disease (Figure 6C).

Discussion

This clinical trial was conducted to determine whether more intensive neoadjuvant androgen deprivation therapy could be obtained with the addition of abiraterone acetate plus prednisone (referred to herein as abiraterone) to leuprolide, to determine the extent to which this could mediate regression of primary PCa in men with intermediate to high risk disease, and to identify mechanisms that may be contributing to therapy resistance. As reported previously, the addition of abiraterone to leuprolide effectively reduced intraprostatic androgens and a small number of complete or near complete responses were obtained, but substantial residual tumor was found in most cases (9). Analysis of these residual tumors by histology and IHC showed that most were adenocarcinoma, NOS, and that the extent of neuroendocrine differentiation was not increased relative to control untreated tumors. Consistent with this finding, nuclear AR was expressed at intermediate-to-high levels in most cases, and transcriptome profiling showed that substantial AR transcriptional activity persisted in the residual tumor foci. This AR activity was positively associated with *AR* and *NR3C1* (GR) mRNA levels, but not with expression of *AKR1C3* that drives increased intratumoral androgen synthesis from adrenal gland precursors in metastatic CRPC. Distinct from mCRPC, we similarly did not observe increased expression of the AR-V7 splice variant, which correlates with resistance to ADT in metastatic CRPC (16). Although this does not preclude expression of other variants we did not measure, it suggests that increased AR splice variant expression is a longer term adaptation or there is less selective pressure favoring their expression in the prostate versus at metastatic sites.

Significantly, AR transcriptional activity was not associated with increased proliferation (and was instead negatively correlated), indicating that AR reactivation beyond some basal levels did not provide a clear growth advantage for residual tumor cells, and consistent with the apparent lack of selection for increased intratumoral androgen synthesis. Interestingly, analyses of primary PCa and metastatic CRPC data sets similarly indicate proliferation is not associated with increased AR signaling, with a negative correlation in some cases (23). The somewhat more robust negative correlation in these neoadjuvant cases versus metastatic

CRPC may in part reflect more dependence for proliferation on AR-regulated growth factors from prostatic stromal cells, which in turn are also impacted by ADT. Moreover, in the absence of growth factors or other factors driving proliferation, the AR in residual tumor cells with higher AR activity may be driving metabolic pathways related to differentiated functions, causing endoplasmic reticulum stress and decreasing proliferation.

In contrast to AR activity, increased proliferation in residual tumors was associated with decreased *RB1* mRNA. Prior studies showed that pRb inactivation could circumvent the G1/S block mediated by androgen deprivation (38) and was associated with progression to CRPC after castration therapy (39), and *RB1* loss is emerging as a mechanism of resistance in advanced CRPC after treatment with abiraterone and enzalutamide (18,40–42). Interestingly, *RB1* loss in these latter advanced tumors is associated with NED (25,43,44), but was not associated with NED in these neoadjuvant treated tumors. Significantly, we found deep deletions to *RB1* in 4 foci (and shallow deletions in another 9 foci), which is significantly greater than the incidence in the TCGA data set of primary PCa (21), but comparable to the rate of homozygous *RB1* inactivation in mCRPC (~9%) (18). These deep *RB1* deletions we observed were associated with decreased *RB1* mRNA, but some foci with relatively low *RB1* mRNA did not have apparent *RB1* losses. Focal genomic *RB1* losses that are not apparent based on segmented WES read depth may be occurring in these latter cases, although epigenetic mechanisms also could be contributing to decreased *RB1* expression. In any case, these findings suggest that subclonal *RB1* loss may be more common than previously appreciated in intermediate to high risk primary PCa, and may be an earlier event, independent of NED, in the development of initial resistance to aggressive androgen deprivation therapy (see Fig. 6C).

Recent studies from others and us have clearly shown that primary PCa, like many other cancers, can have extensive microheterogeneity (12,45–49). However, the contribution of this microheterogeneity in the primary tumor to metastatic disease and therapy resistance has not been clear. Significantly, in addition to *RB1* losses, we identified multiple established or likely oncogenic alterations that were present in only one of two tumor foci analyzed. This indicates that they were subclonal in the tumor prior to therapy, and that they likely contributed to the persistence and expansion of the corresponding tumor focus. In men who relapse after RP, we suggest that these foci with subclonal oncogenic alterations may be the origin of tumor cells that give rise to metastatic CRPC. This hypothesis is being tested in long term follow up studies.

Significantly, as noted above, AR activity was negatively correlated with proliferation, and was found to be similarly inversely associated with proliferation in a recent study of advanced mCRPC (23). Nonetheless, it is important to note that substantial AR expression and activity persisted in most foci of residual tumor, and similarly persists in most cases of mCRPC (1,50). Therefore, while this AR activity clearly is not sufficient to drive proliferation, and higher levels may suppress proliferation, it may nonetheless be necessary for tumor cells to maintain some basal level of AR activity in order to survive. Moreover, AR in metastatic foci may be driving expression of growth factors that are stromal derived in the primary tumors, making the metastatic tumors more dependent on intrinsic AR activity. Hence, our observations are consistent with the recently reported LATITUDE (51) and

STAMPEDE (52) studies, in which survival benefits were observed for the earlier treatment of hormone sensitive metastatic PCa with abiraterone. In the case of localized disease, we hypothesize that more robust responses still might be obtained through therapies that further ablate AR activity, although it is not clear whether even complete AR annihilation would be effective in tumors that have lost *RB1*. The efficacy of further AR suppression is currently being studied in trials that combine neoadjuvant leuprolide and abiraterone with direct AR antagonists.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Significance

Neoadjuvant androgen deprivation therapy for prostate cancer selects for tumor foci with subclonal genomic alterations, which may comprise the origin of metastatic castration-resistant prostate cancer

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Figure 1. Reduced proliferation and AR pathway IHC markers in trial cases

(A) A representative trial case (case 513) demonstrating Ki-67 at very low (left), low (middle), and high (right) levels in three tumor foci of the same index tumor; CK18 and Ki-67 dual IHC stain ($\times 400$ magnification). Brown chromogen: Ki-67. Red chromogen: cytokeratin 18 (CK18). (B and C) Box-and-whiskers plots of Ki-67 indices comparing treated to untreated cases (B) and 12-week and 24-week treatment arms (C). For (B) and (C), the box depicts the 25th percentile, median, and 75th percentile, and whiskers depict the lowest value and the 75th percentile + 1.5 $\times$ the interquartile range (IQR). Open circles depict outlier Ki-67 index values greater than the 75th percentile + 1.5 $\times$ the IQR. Difference

between 12- and 24-week arms was not significant (n.s.) at $P = 0.3481$. (D, G, H, I) Stacked bar chart of nuclear AR IHC scores (D), AR-V7 IHC scores (G), PSA IHC scores (H), and NKX 3.1 IHC score (I). For the 12- and 24-week treatment arms, the difference was not significant at $P = 0.7998$ (AR), $P = 0.2033$ (AR-V7), $P = 0.1670$ (PSA), and $P = 0.1413$ (NKX 3.1). For the treated and untreated cases, $P = 0.4625$ (AR-V7). (E) *In situ* hybridization staining of a representative trial case (case 523) depicting low (left) or high (right) levels of AR-V7 (top) or total AR (bottom) transcript in two tumor foci of the same index tumor ($\times 400$ magnification). (F) IHC demonstrating AR-V7 at very low (left), intermediate (middle), and high (right) levels in three foci of case 523 ($\times 400$ magnification). All statistical differences in (B), (C), (D), (G), (H), and (I) were determined using the Wilcoxon rank sum test.

Figure 2. Oncogenic alterations in residual tumors revealed by IHC staining

(A) Representative IHC of trial case 601 depicting complete negative staining of PTEN in tumor cells ($\times 400$ magnification). (B) Consecutive section of the same tumor depicting increase in phosphorylation of Akt at Serine 473 ($\times 400$ magnification). (C and D) Correlation between PTEN loss scores and pAkt (C) ($N = 47$) and Ki67 (D) ($N = 46$) in trial cases. Each open circle represents a single case at intervals of 0, 1, 2, and 3 for PTEN loss score on IHC versus 0, 1, 2, and 3 for pAkt weak, moderate, and strong intensity (C), or versus Ki67 index at 0–100% on IHC (D). PTEN loss score measures the extent of tumor cells with PTEN loss (negative staining). Spearman's ρ (r) rank coefficient is shown with

the 95% confidence interval. (E) Representative IHC of trial case 513 depicting focal pRb loss ($\times 400$ magnification). pRb-positive region marked with straight hash lines. pRb-negative region marked with dots.

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Figure 3. Transcriptome based analysis of AR activity and proliferation

(A) Unsupervised hierarchical clustering of the 1000 genes with the most variance across the entire dataset using Ward's method distinguished the source of samples between treated and untreated tumors. In most cases, foci from the same individual had lower within-cluster variance. (B,C) Box-and-whiskers plots of 131-gene proliferation signature (19) ssGSEA score comparing treated to untreated cases (B) and 12-week and 24-week treatment arms (C). Difference in (B) was not significant at $P = 0.1543$. Open circle in (C) depicts outlier ssGSEA score of 513.D16, which was greater than the 75th percentile plus 1.5 $\times$ the interquartile range (IQR). (D, I, J) Correlation of the $\log_2$ -transformed microarray

expression value for *RB1* from each microdissected focus with the 131-gene proliferation ssGSEA score (D), 267-gene AR activity ssGSEA score (I) and 80-gene *RB1* loss score (J). Sample 513.D16 is omitted from (D) due to outlier high ssGSEA proliferation. (E, F, G) Box-and-whiskers plots of 267-gene AR activity signature (20) ssGSEA score comparing treated to untreated cases (E), comparing the treated group to the Prostate Cancer Foundation-Stand up to Cancer CRPC group (18) and the neuroendocrine variant prostate cancer (NEPC) group (40) (F), and comparing the 12-week and 24-week treatment arms (G). Difference between treated group and CRPC was not significant (n.s.) at $P = 0.5651$, and the difference in (G) was not significant at $P = 0.0534$. For (B), (C), (E), (F), and (G), ssGSEA scores were plotted using Tukey's method for a box-and-whiskers plot, where the box depicts the 25th, 50th, and 75th percentiles, whiskers depict either the lowest and highest values or the 25th and 75th percentiles $\pm 1.5 \times \text{IQR}$, and open circles depict outlier scores higher or lower than the 75th/25th percentile $\pm 1.5 \times \text{IQR}$. (H) Correlation of the 267-gene AR activity ssGSEA score and the 131-gene proliferation ssGSEA score from each microdissected focus, except for 513.D16, which had outlier high ssGSEA proliferation. Statistical differences in (B), (C), (E), (F), and (G) were determined using the t test with Welch's correction, and Pearson r correlation coefficients are shown for (D), (H), (I), and (J) with their 95% confidence intervals.

Figure 4. Pathways altered in residual tumor and upstream regulators

(A) Volcano plot depicting differentially expressed genes in the treated versus untreated cases, with the adjusted $-\log_{10} P$ value on the y -axis and the $\log_2$ -transformed fold change on the x -axis. The majority of significantly differentially-expressed genes were down-regulated (blue dots) in the treated versus untreated cohort. Upregulated genes are depicted with red dots. (B) Statistically significantly up- and down-regulated genes were used as the basis for deriving enriched pathways using Ingenuity Pathway Analysis. $-\log_{10} P$ value is shown for the top 10 pathways. (C) Ingenuity Regulatory Analysis was used to identify key regulators that are significantly activated (orange) and inhibited (blue), estimated based on gene expression from the significantly differentially expressed genes depicted in (A). (D)

Correlation of the 267-gene AR activity ssGSEA score and $\log_2$ -transformed microarray expression value for *XBPI* from each microdissected focus. Pearson r correlation coefficient is shown with its 95% confidence interval.

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Figure 5. Shared and unique SCNVs and oncogenic alterations in subset of residual tumor foci
 (A) Depiction of genome-wide copy number alterations derived from segmented exome sequencing data. Gains are depicted in red; losses are shown in blue. Cases are grouped by focus for the first 15 patients shown. Single foci from 3 patients are shown at the bottom. (B) Euler diagrams depicting shared and distinct somatic genomic events. Circles are drawn to scale for all mutations and copy number alterations that passed filtering requirements. Annotations are limited to alterations overlapping with 727 curated cancer-related genes (12). Cases 004, 505, 508, 513, 516 and 518 are shown.

Figure 6. Enrichment of biallelic alterations in residual tumor foci

(A) Manhattan plot showing the selective enrichment by Fisher's exact test for biallelic events to genes in the neoadjuvant abiraterone study verses in untreated primary PCa from the TCGA study (21). A gene was counted if two somatic events were reported (such as two somatic mutations or a single copy loss plus a somatic mutation to the remaining copy). A deep deletion was counted as two somatic events. The horizontal dotted line is drawn at $-\log_{10}$ for $P = 0.05$. Each dot size is drawn proportional to the number of cases in the neoadjuvant abiraterone study with biallelic events to the given gene. Genes are arranged by chromosome (autosomes only), with red dots indicating genes with biallelic events with a greater proportion of cases in the neoadjuvant abiraterone cohort vs. the prostate TCGA cohort. (B) Negative Spearman correlation ($r = -0.43$; not significant) of $\log_2$ -transformed microarray expression value for *RB1* and the number of *RB1* alleles remaining as estimated

by segmented read-depth copy number analysis. Each dot represents a single focus. (C) Schematic illustration (based on case 505) of the functional impact of genomic and phenotypic changes that are observed following neoadjuvant androgen deprivation therapy that may be relevant for the clinical development of CRPC and progression to metastatic disease. Early shared events in this case included *TP53* loss and single copy losses of *RB1*, *BRCA2*, and *CDKN2A*. Treatment then selects for a subclone with persistent AR activity driven by *PTEN* loss and gain of *MYC* and *AR*, and a distinct subclone with low AR activity and driven by further loss of *RB1* and *BRCA2*. We hypothesize that the former subclone may then give rise to AR driven mCRPC, while the latter subclone may give rise to AR low or negative mCRPC, without or with neuroendocrine differentiation.

Table 1

Clinicopathologic and IHC characteristics of trial cases and matched untreated controls

	Treated (N=49)	Control (N=49)	P Value
Median age at diagnosis (IQR)	58 (54, 63)	63 (57, 68)	0.014 ^a
Race (%)			
Other	44 (89.8)	38 (77.6)	0.308 ^b
African American	5 (10.2)	8 (16.3)	
Unknown	0 (0)	3 (6.1)	
Median ng/ml pre-RP PSA (IQR)	10.0 (5.4, 16.4)	6.5 (5.5, 9.4)	0.109 ^a
Biopsy:			
Grade Group			
GG1 (GS3+3=6)	0	0	0.757 ^b
GG2 (GS3+4=7)	5	6	
GG3 (GS4+3=7)	12	14	
GG4 (GS8)	14	16	
GG5 (GS9–10)	18	13	
Median number of positive cores (IQR)	6 (5, 8)	6 (4, 8)	0.598 ^a
Median cancer volume by % involvement (IQR)	90 (67, 100)	80 (50, 90)	0.104 ^a
RP:			
Median prostate weight in gm (IQR)	33 (25, 38)	46 (40, 58)	0.000 ^a
Grade Group			
GG1 (GS3+3=6)		0	
GG2 (GS3+4=7)		21	
GG3 (GS4+3=7)		9	
GG4 (GS8)		1	
GG5 (GS9–10)		18	
No. of pT stage (%)			
pT2	19 (38.8)	12 (24.5)	0.116 ^b
pT3a	11 (22.4)	20 (40.8)	
pT3b	19 (38.8)	17 (34.7)	
No. of pN stage (%)			
pN0	40 (81.6)	42 (85.7)	0.585 ^b
pN1	9 (18.4)	7 (14.3)	
Margin status (%)			
Negative	41 (83.7)	32 (64.0)	0.026 ^b
Positive	8 (16.3)	18 (36.0)	
PTEN IHC (%)			
Loss	23 (46.9)	25 (51.0)	0.838 ^b
No loss	24 (49.0)	24 (49.0)	
Unknown	2 (4.1)	0 (0)	

	Treated (N=49)	Control (N=49)	P Value
ERG IHC (%)			
Negative	31 (62.0)	28 (57.1)	0.536 ^b
Positive	18 (36.0)	21 (42.9)	

GG, Grade group; GS, Gleason score; IQR, interquartile range; PSA, prostate-specific antigen

^aWilcoxon rank sum test;

^bPearson chi-square test