Impact of therapy on genomics and transcriptomics in high-risk prostate cancer treated with neoadjuvant docetaxel and androgen deprivation therapy

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Support: Research reported in this publication was supported by NIH 3U10CA180821-03S3 (H.B., A.W., S.H., M.E.G), Department of Defense PC121341 (H.B.), NIH 5U01CA157703 (S.H., M.E.G), Canadian Cancer Trials Group through grants from the Canadian Cancer Society Research Institute (#021039 and #704970) and NIH (#CA077202 and #CA180863), and an
Alliance for Clinical Trials in Oncology Clinical Scholar Award in honor of Dr. Emil “Tom” Frei, III (H.B.).

Running title: Molecular analysis of high risk PCA after neoadjuvant docetaxel + ADT

Key words: high-risk prostate cancer, neoadjuvant, docetaxel, ADT, genomics, integrative molecular analysis

Figures: 4
Tables: 1
References: 45
Supplemental files: 2
Abstract

Background: The combination of docetaxel chemotherapy and androgen deprivation therapy (ADT) has become a standard treatment for patients with metastatic prostate cancer. The recently accrued Phase III CALGB 90203 trial was designed to investigate the clinical effectiveness of this treatment approach earlier in the disease. Specimens from this trial offer a unique opportunity to interrogate the acute molecular response to docetaxel and ADT and identify predictive biomarkers.

Methods: We evaluated baseline clinical data, needle biopsies and radical prostatectomy (RP) specimens from 52 (of 788) patients enrolled on CALGB 90203 at one high volume center. Pathology review, tumor and germline targeted DNA sequencing (n=72 genes), and expression profiling using Nanostring platform (n=163 genes) were performed to explore changes in critical prostate cancer pathways linked to aggression and resistance.

Results: 3/52 patients had only microfocal residual cancer at prostatectomy. The most common alterations included TMPRSS2-ERG fusion (n=32), TP53 mutation or deletion (n=11), PTEN deletion (n=6), FOXA1 (n=6), SPOP (n=4) mutation, with no significant enrichment in post-treated specimens. We did not observe AR amplification or mutations. The degree of AR signaling suppression varied among treated tumors and there was up-regulation of both AR and AR-V7 expression as well as a subset of neuroendocrine and plasticity genes.

Conclusions: These data support the feasibility of targeted and temporal genomic and transcriptome profiling of neoadjuvant-treated prostate cancer with limited formalin-fixed paraffin embedded tissue requirement. Characterization of the heterogeneity of treatment response and molecular outliers that arise post-treatment provides new insight into potential early markers of resistance.

Statement of Translational Relevance

Recent phase III trials have positioned chemohormonal therapy (docetaxel and androgen deprivation therapy (ADT)) as a standard treatment option for patients with advanced hormone naive prostate cancer. There are no available molecular biomarkers to identify which patients are most likely to benefit and limited data exploring early mechanisms of treatment resistance. In the current study, we demonstrate the feasibility of molecular analyses of pre-treatment diagnostic biopsies and post-treated prostatectomy specimens from patients enrolled on a phase III trial of neoadjuvant docetaxel and ADT. Through a multi-level combination of pathology, DNA sequencing and transcriptome profiling, we offer insights into the acute molecular response to chemohormonal therapy and potential strategies to identify predictive biomarkers.
Introduction

Prostate cancer arises as an androgen-driven disease, dependent on ligand-activation of the androgen receptor (AR) for growth and maintenance (1). Consequently, systemic therapies for advanced prostate cancer have focussed on inhibition of AR signaling through reduction of available ligand via androgen deprivation therapy (ADT) or direct antagonism of the AR receptor (2). Even taxane chemotherapies, such as docetaxel, are thought to be effective at least in part through mechanisms that lead to disruption of AR signaling (3,4). Recent randomized phase III trials have shown that combining docetaxel with ADT for patients with newly diagnosed metastatic hormone-sensitive prostate cancer provides a significant overall survival benefit when compared with ADT monotherapy (5-7).

A rational next question is whether the same benefit may be observed if these therapies were applied earlier in the disease. The multicenter phase III Cancer and Leukemia Group B (CALGB) 90203 trial, “A Randomized Phase III Study of Neo-Adjuvant Docetaxel and Androgen Deprivation Prior to Radical Prostatectomy Versus Immediate Radical Prostatectomy in Patients with High-Risk, Clinically Localized Prostate Cancer”, was designed to evaluate this question by studying ADT plus docetaxel in patients with newly diagnosed high-risk clinically localized prostate cancer in the neoadjuvant setting. The primary clinical endpoint is to determine if neoadjuvant therapy decreases 3-year recurrence rates when compared with radical prostatectomy (RP) alone. Patient accrual completed in 2015 and the trial results are expected to be reported in 2018. CALGB is now part of the Alliance for Clinical Trials in Oncology.

Tissue specimens collected from the CALGB 90203 study provide an unprecedented opportunity to understand treatment response and early mechanisms of treatment resistance. This may ultimately elucidate biomarkers to help identify patients most likely to benefit from the combination of ADT and docetaxel. Regardless of the outcome of the CALGB 90203 trial primary endpoint, this biomarker information may also be relevant for the significant proportion of prostate cancer patients with metastatic disease now being treated with standard of care chemohormonal therapy and may ultimately help improve patient selection. Molecular biomarker development for this population will require the development of tools amenable to small amounts of tissue from formalin fixed diagnostic tissue biopsies.

In this proof of principle study, we posited that resistant pathways observed in metastatic castration-resistant prostate cancer (CRPC) may be detected early in a subset of patients treated with short-term docetaxel and ADT and this is associated with the presence of treatment resistant cells. Recent metastatic tissue-based studies combined with preclinical modeling have illuminated diverse mechanisms of resistance to AR-directed therapies (8-10) and to docetaxel in the CRPC setting (11-15). Tissue analyses from patients with intermediate and high-risk prostate cancer enrolled in prior phase II trials of neoadjuvant ADT with or without potent AR pathway inhibitors have elucidated changes in AR signalling and correlated the presence of intra-prostatic tumor androgens and treatment-induced adaptive response pathway changes with residual disease (e.g., including pathologic complete remission (pCR) and near pCR rates) (16-19). We previously reported in the CUOG-PO1a trial that two of 64 patients treated with...
neoadjuvant docetaxel plus androgen ablation for 24 weeks had a complete pathological response (no tumor in final pathological specimen) (20). While complete eradication of cancer following neoadjuvant therapy is rare, pathological specimens often have marked tumor regression that creates challenges in molecular characterization of small residual foci of cancer.

We aimed to develop molecular tools amenable to small amounts of tissue in order to dissect the genomic and transcriptomic landscape of tumors before and after therapy from patients enrolled on CALGB 90203. We hypothesized that 1) the combination of AR targeted therapy and docetaxel shapes changes in the molecular landscape of early, aggressive prostate cancer; and 2) that tumor clones present after therapy may represent either intrinsic or early acquired-resistance.

Materials and Methods

Clinical cohort

Patients with newly diagnosed high-risk, clinically localized prostate adenocarcinoma were enrolled on the Phase 3 CALGB 90203 (Alliance) trial from 2006 to 2015 across the United States and Canada (Figure 1A). Entry criteria and summary of trial endpoints are described in Supplementary Information. Patients in trial arm A received six cycles of docetaxel at a dose of 75 mg/m² administered every 3 weeks in combination with a luteinizing hormone-releasing hormone (LHRH) agonist for 18-24 weeks, followed by RP. Arm B consisted of patients undergoing standard RP without neoadjuvant chemo-hormonal therapy. A total of 788 patients were enrolled. The primary endpoint is 3-year biochemical PFS and is expected to be completed in 2018. For the current study, baseline clinical data, baseline needle biopsies and RP specimens from 52 of these patients (28 on arm A; 24 on arm B) enrolled at one high volume center (University of British Columbia (UBC)-Vancouver General Hospital) were evaluated (with approval from the UBC Ethics Board and the Alliance Genitourinary Committee). Clinical data and pathology were retrieved locally without access to Alliance central database or biorepository. Each participant signed an IRB-approved, protocol-specific informed consent in accordance with federal and institutional guidelines.

Pathologic evaluation

Formalin-fixed paraffin embedded (FFPE) diagnostic core biopsies and fresh frozen (FF) and FFPE tissue blocks from RP specimens were reviewed by the study pathologist (L.F.) for tumor size, Gleason grade, and other morphologic features. Samples were excluded from analysis if insufficient tumor was present due to low cellularity or unavailability of tumor blocks. Overall treatment effect was scored as 0-3 defined by the severity of six morphologic characteristics (Supplementary Methods, Figure 1B) (21,22). Representative tumor dense areas and adjacent benign tissue were selected for macrodissection and DNA/RNA extraction (Supplementary Methods).
DNA sequencing

We employed targeted sequencing of FFPE needle biopsies and RP specimens using a custom NimbleGen SeqCap EZ Choice Library and Illumina machines. Our custom design includes 72 prostate cancer relevant genes, including AR pathway genes (e.g., AR, FOXA1, TMPRSS2) and other common prostate cancer alterations including TP53, SPOP, cell cycle genes (e.g., CCND1, RB1), DNA repair genes (e.g., BRCA1/2, FANC family genes, ATM, MSH2/6), and PI3K pathway genes (e.g., PIK3CA, PTEN) (Supplementary Table 1). Details regarding DNA extraction and sequencing are described in the Supplementary Methods.

Somatic mutations were identified by comparing allele read counts between tumor and adjacent benign samples. We required somatic mutations to be supported by at least 10 ‘mutant’ reads, and to have a mutant allele fraction of at least 2.0%, at least 2x higher than the allele fraction in the paired normal samples, and at least 20x higher than the background error rate at the site. Mutations in low mappability regions of the genome were discarded. Variant effects were predicted using ANNOVAR version 2016-02-01. Each variant’s frequency in the KAVIAR, ExAC and COSMIC databases was annotated using in-house software. All mutation candidates were manually reviewed for indel-related alignment artifacts, homologous regions, high background error rates, and positional bias using the Integrative Genomics Viewer. Two patients lacked a matched benign tissue sample, and were manually reviewed for mutation candidates of potential germline origin.

To call copy number changes, aligned reads were counted in all captured genome regions using bedtools-2.25.0(23). GC fractions were calculated for all target regions, and Loess regression was applied to bait logratios to correct for GC content bias. All Loess fits were visually validated for soundness. Coverage logratios were calculated between each sample and a consensus reference representing the median of all normal samples. Log ratios were corrected for sample-specific differences in overall coverage using median-of-ratios normalization across target regions in chromosomes 2, 4, 12, 14, 15, 19 and 20 (selected for their low rate of copy number alterations in late-stage prostate cancer). All paired normal samples were searched for heterozygous germline SNPs with good coverage, and their allele fractions were calculated in the corresponding tumor samples. A median heterozygous SNP allele fraction 0.5 + median (abs(AF - 0.5)) was calculated for every gene in each tumor sample.

Gene Expression Profiling

We developed a targeted gene panel for this study using the NanoString nCounter® that was applied to FFPE biopsy and RP tissues with limited RNA input requirement (<300ng). The custom gene panel included 163 genes chosen based on their known or potential role in prostate cancer progression, including AR and AR signaling genes (24), the AR V7 splice variant, EMT/plasticity and neuroendocrine prostate cancer associated genes (10), cell cycle, WNT, PI3K/AKT pathway genes, TMPRSS2-ERG fusion transcript, and control and housekeeper genes (Supplementary Table 2). An AR signaling score was quantified for each case using AR pathway genes (24) and methodologies previously described (10) based on correlation with the LNCaP cell line as a reference sample known to have active AR signaling.
Details regarding RNA extraction and processing are described in the Supplementary Methods.

Raw count data was normalized using the nSolver™ analysis software version 2.0, which normalizes samples according to positive and negative control probes and the geometric mean of the 6 housekeeping primers. RNA from fresh/frozen specimens was also used when available for QC and comparative analysis and for RNA-seq when feasible (using protocols previously described (10)). The edgeR package was used to determine genes that were differentially expressed when comparing the treated and untreated cases. To take advantage of the platform design, which includes negative, positive and housekeeping controls, the normalized counts were used as input measurements for the edgeR library. The negative binomial method was used to identify differentially expressed genes between the treated and untreated cases. The differentially expressed genes were identified by fitting a generalized linear model on the set of 45 RPs, comparing specimens between the two arms. Hierarchical clustering and principal component analysis (PCA) with coefficient of variation (BCV) in edgeR library package distance was used.

Results

Baseline clinical and pathological features

Baseline clinical characteristics for the 52 patients evaluated in this study are summarized in Table 1 and Supplementary Table 3. Median baseline serum PSA was 11 ng/ml (range 6.3-19.8 ng/ml) and 88% of patients had a Gleason score ≥8, reflective of high-risk disease and consistent with the trial eligibility criteria. There were 20/52 patients who received no treatment prior to surgery, and 24/52 who received combined docetaxel plus ADT (Table 1 and Figure 1C). A further 8/52 received ADT alone due to patients in the treatment arm (n=4) declining chemotherapy or withdrawing after randomization, and patients in the non-treatment arm (n=4) receiving 1-3 months of neoadjuvant ADT (allowed as part of the protocol). Patients receiving docetaxel plus ADT had a higher serum PSA at diagnosis compared to those receiving no treatment (median PSA 13 vs 7.6 ng/ml). However, as expected, pre-surgery median PSA levels decreased in the docetaxel plus ADT group (0.4 vs 9.1 ng/ml), consistent with biochemical response to therapy.

Representative photomicrographs of pathology review on tumor cellularity and treatment effect are shown in Figure 1B and Supplementary Figure 1. There were 49/52 RPs (94%) with sufficient tumor and matched normal material for DNA extraction (3/52 had near complete pathologic responses), which was successful in all 49 cases (median yield = 1450 ng). Of these, mRNA was successfully extracted and met quality control standards for Nanostring in 45 cases (median 265 ng RNA).

Although 58% of patients had their diagnostic biopsies (prior to study enrollment) performed outside Vancouver General Hospital, we were able to obtain FFPE biopsy blocks for 49/52 patients. Based on pathology review, 47/49 (96%) of diagnostic biopsies had sufficient tumor
material for DNA and RNA extraction. DNA extraction was successful in 44/47, with a median yield of 28 ng. Thirty-four needle biopsies met QC standards for Nanostring analysis (median 255 ng RNA). Analysis of both DNA and RNA of matched biopsy and RP pairs was feasible for 34/47 (81%, 95% CI= 67%-91%) patients (Figure 1A,C).

The dates of subject enrollment ranged between 2006 and 2015. The median age of tumor biopsy tissue specimens at time of molecular analysis for this study was 66 months (range 7-97 months) and the median age of RP specimens was 61 months (range 5-86 months).

Differences in mutational landscape before and after neo-adjuvant treatment

We performed targeted DNA sequencing across a panel of 72 known prostate cancer genes (Supplementary Table 1). Median on-target sequencing depth in the RP specimens was ~500X. Predictably, the archival biopsy specimens gave more variable data including a high frequency of PCR duplicates and reduced target region coverage (Supplemental Figure 2). Although this variability did not affect mutation calling, there were inherent biases in copy number profiles that were not explained by GC fraction of target regions, extracted DNA yield, or systematic differences between biopsy and prostatectomy samples. Therefore, we excluded biopsy copy number calls from global analyses.

Forty two out of 52 (81%) patients had DNA sequencing performed on paired biopsy and RP specimens, with 9 of the remaining 10 patients having either biopsy or RP data alone. Minimum tumor cell fractions from targeted DNA sequencing are reported in Supplementary Table 5. Across the cohort, we detected a total of 106 somatic mutations, including 57 predicted to cause coding changes (18 were truncating mutations) (Supplementary Table 5). Coding somatic mutations were consistent with aggressive primary prostate cancer (25), including deleterious mutations involving FOXA1 (n=6), SPOP (n=4), PTEN (n=6) and KMT2C (n=6) (Figure 2, Supplementary Figure 3). Eleven patients (22%) had pathogenic mutations in TP53 (compared to TCGA frequency of 59/498 (12%)). Three out of 52 patients harbored truncating germline mutations involving DNA damage repair genes BRCA2 (n=2) or ATM (Supplementary Table 6). Whole exome sequencing of adjacent FFPE sections and/or fresh/frozen tissue blocks from 20 RP cases confirmed mutation calls from targeted sequencing and did not reveal any additional mutations across the 72 genes in our panel (Supplementary Table 7).

Among the diagnostic biopsies evaluated, 35/44 (80%) had ≥1 somatic mutation, with 23/44 (52%) having ≥1 non-synonymous mutation (Figure 2, Supplementary Figure 3). This is consistent with recent TCGA data where 159/333 (48%) of primary prostate cancer had ≥1 non-synonymous mutation in the 72 genes across our panel (25). Although 85% of the pre-treatment biopsies from patients receiving subsequent neo-adjuvant therapy had mutations, after treatment (i.e. in RP specimens) this figure was 59% (in untreated patients this comparison was 75% vs 71%). The average number of detected somatic mutations was not lower in treated compared to untreated RP specimens (1.0 vs 1.55) (Supplementary Figure 4).
mutant/variant allele frequency (MAF) was significantly lower in post-treated RP specimens (mean = 13.4%) compared to either pre-treatment biopsy (mean = 24.1%; t-test) or untreated RP specimens (mean = 24.7%) (Supplementary Figure 4). This trend was particularly evident amongst paired mutations (i.e. those shared by matched biopsy and RP), where 95% of mutations in post-treatment RP specimens had a lower MAF than in the matched pre-treatment biopsy, compared to only 33% in the untreated specimens (mean [median] fold change treated vs untreated: 0.47 [0.40] vs 2.02 [1.13] (Supplementary Figure 4). Overall these data are consistent with a variable reduction in tumor cellularity/burden post-treatment and raises the possibility of reduced tumor clone diversity in the wake of potent chemo-hormonal therapy. Despite this decrease in mutational detection post-therapy, there were significant tumor cell intrinsic gene expression changes detected across the cohort as described below.

Recent metastatic tissue biopsy studies have suggested that some gene mutations are enriched in CRPC compared to primary treatment-naive prostate cancer. For example, TP53 mutations are found in 46.7% of CRPC but only 12.2% of primary tumors, while mutations in the AR ligand binding domain are present in 20-25% of CRPC but absent from untreated prostate cancer (8,25). The latter are a mechanism by which prostate cancer cells become resistant to ADT. Among the RP specimens exposed to neo-adjuvant therapy, individual gene mutation frequencies were not high enough to detect a statistically significant trend post-treatment (e.g. consistent enrichment or depletion). However, five TP53 mutations were detected in the post-treated specimens (Supplementary Figure 5). Two patients with pathogenic FOXA1 mutations in their pre-treatment biopsy had an apparent pathologic complete response in the RP specimen (and no tissue available for sequencing). We did not detect any AR mutations in post-treated RPs (at >1% MAF), suggesting that 4.5 months of ADT is not sufficient time to yield a large population of AR mutant clones.

Copy number alterations in the untreated RP specimens were consistent with high-risk disease, including frequent PTEN (8/22) and RB1 (6/22) loss (examples in Supplementary Figure 6). However, there were some unusual events including a deep deletion of ZBTB16 in the biopsy and RP specimen from one patient (case 5346), and an amplification of CDK6 in both the biopsy and RP specimen from another patient (case 6803) (Supplementary Figure 6). Homozygous deletions of the transcriptional repressor and putative tumor suppressor ZBTB16 (PLZF) were recently identified in CRPC (8,26).

Evidence of treatment-induced transcriptome changes

Gene expression levels of the panel of 163 genes and a list of differentially expressed genes by univariate analysis are summarized in Supplementary Tables 8-11. For seven RP specimens with sufficient fresh/frozen tissue for both Nanostring and RNA-seq, gene expression was compared and showed high correlation (average Pearson’s correlation coefficient= 0.85) (Supplementary Figure 7); Nanostring from matched FFPE and fresh/frozen tissue were also compared and showed high correlation (Pearson correlation coefficient= 0.96) (Supplementary Figure 8). Copy number losses, such as PTEN (detected by DNA sequencing), were associated with lower expression at the mRNA level (Supplementary Figure 9). TMPRSS2-
ERG fusion transcript was detected in 32 cases and associated with ERG mRNA overexpression (Pearson’s correlation coefficient =0.8, Supplementary Figure 10).

Unsupervised analysis of mRNA data of RP specimens of all the genes revealed clustering of treated and untreated cases (Supplementary Figures 11-13). Differentially expressed genes are depicted in Figure 3 and reported in Table S8. Molecularly distinct subsets were identified in both groups and were not explained by differences in tumor cellularity (Supplementary Figure 13). When comparing tumors with high minimum tumor cellularity to those with low (or unknown) tumor cellularity from targeted DNA sequencing, we also did not observe large differences in the levels of relatively tumor cell intrinsic mRNA transcripts such as AR, KLK3 or ERG (Supplementary Figure 14). The heterogeneity observed is supportive of a likely lack or minimal contribution of batch effect or poor signal driving changes and likely driven by biologic differences amongst patients. In the neoadjuvant treatment arm, the majority of the evaluated genes were significantly upregulated compared to untreated RP cancers (Supplementary Table 8, Supplementary Figure 12).

As expected, there was significant downregulation of the AR-target gene KLK3 (PSA) expression in post-treated RP specimens concordant with an overall decline in serum PSA (Figure 4, Supplementary Figure 15) though the degree of suppression of AR signaling genes and AR score in treated cases varied (Supplementary Figures 16-17). Expression of both AR and AR-V7 transcripts were higher in the treated group (Figure 4) and strongly correlated; the ratio of AR-V7 to wild type AR was similar to the untreated cases (Supplementary Figure 18). Although baseline PSA was higher in the treated subgroup, there were no significant differences in baseline biopsy tissue PSA (KLK3) expression to explain differences in post-treatment AR signalling (Supplementary Figures 19-20). We also observed significant up-regulation of other hormone receptors (ie., estrogen receptor (ESR1), progesterone receptor (NRC3C), glucocorticoid receptor (NRC31)) in post-treated specimens (Figure 3). These observations are potentially explained by residual AR activity and/or activity of alternative transcriptional activators (ie., AR bypass mechanisms).

There was higher gene expression of a subset of plasticity and neuroendocrine lineage genes in post-treated specimens including classical neuroendocrine markers such as CHGA (chromogranin A) (Supplementary Figure 21). PEG10 (Paternally Expressed 10), a retrotransposon-derived gene up-regulated during neuroendocrine trans-differentiation and functionally linked to Rb and p53 (27), was also higher in treated cases (Supplementary Figure 22). The SPDEF gene, recently found to be down-regulated in metastatic castration resistant neuroendocrine prostate cancer due to DNA methylation (10) was also significant downregulated in the treated vs. untreated specimens from this cohort (Supplementary Figure 23). SPDEF is an ETS family transcription factor, previously described as a regulator of cellular differentiation and suppressor of tumor metastasis through inhibition of epithelial mesenchymal transition (EMT) (28). Treatment-induced up-regulation of neuroendocrine markers including PEG10 and downregulation of SPDEF are hypothesis generating and suggest that plasticity may occur after short-term therapy. The frequency and prognostic value of these markers and
their relationship with other lineage plasticity associated genomic alterations such as TP53 and RB1 (29-31) and MYCN (32,33) will be elucidated with more cases and extended follow-up.

Surprisingly, there was one significant outlier case in the untreated arm with very high neuroendocrine marker expression (Figure 3). These findings prompted re-review of the pathology for this case, and the tumor was confirmed by morphology to be a high grade neuroendocrine carcinoma (Supplementary Figure 24) with diffusely positive staining for neuroendocrine markers by immunohistochemistry. These findings highlight the capability of the platform in identifying histologic variants based on molecular features and support the evaluation of pre-treatment biopsies in neoadjuvant studies for the presence of pre-existing changes. We therefore also compared patient-matched prostate needle biopsies with prostatectomy samples in individual patients (n=34). As expected, the pre-treatment biopsies and post-treated specimens showed significant clustering in the treated arm (Supplementary Figure 25) with no significant differences observed in the untreated arm (Supplementary Figure 26). Relative fold changes in gene expression and differentially expressed genes between patient matched pre-treated and post-treated specimens are shown in Supplementary Figures 27-28, which demonstrated heterogeneity in the degree of up-regulation of neuroendocrine markers and down-regulation of AR-target genes including the TMPRSS2-ERG fusion transcript (Supplementary Figures 29-30). Notably the degree of suppression of AR-target genes such as PSA (KLK3), NXK3.1 and TMPRSS2 between matched needle biopsies and neoadjuvantly treated RP specimens significantly correlated with the degree of serum PSA decline in patients before and after neoadjuvant therapy (eg., Pearson’s R = 0.36 for KLK3; R=0.43 for TMPRSS2), in contrast to a negligible correlation observed between biopsies and RP specimens in untreated patients (Figure 4B, Supplementary Figure 31).

Integrative analysis of outlier cases

Radical prostatectomy specimens of two patients (9277 and 8718) that received combined chemohormonal therapy showed less evidence of therapy effect than the rest of the cohort. Both cases had Gleason score 10 at diagnosis, and after neoadjuvant therapy both had nadir pre-surgery serum PSA levels >1 ng/ml as well as significant residual disease by pathology with high Ki67 proliferative index (Supplementary Figure 32). Unlike other treated cases, there was no difference in mutation allele frequency between the pre-treatment biopsy and post-treated RP specimens (Figure 2). Case 9277 had a genomic landscape more consistent with advanced prostate cancer, including TP53 mutation, bi-allelic APC mutation, and PTEN, CDKN1B and RB1 copy number loss (Supplementary Figure 32). Notably there was also evidence of a copy number gain at the AR locus (not present in the pre-treatment biopsy from 9277), and the post-treated specimen of this case had among the highest AR expression compared to the rest of the cohort. Furthermore, gene expression clustering also demonstrated that this case was a unique outlier (Figure 3). When comparing baseline pre-treatment and post-treated specimens for case 8718, AR mRNA expression was high at baseline and increased over two-fold after therapy. Furthermore, based on expression profiles, 8718 clustered closer to the untreated specimens rather than the bulk of the treated tumors (Figure 3). Conversely in a third case (5917), also a patient with a high-grade tumor (Gleason 9) at diagnosis, and high VAF mutations
involving PTEN and FOXA1 in his pre-treatment biopsy, he had a complete pathologic response in his RP specimen such that no tumor tissue was available for macrodissection.

Discussion
The CALGB 90203 study is a multi-institutional phase 3 clinical trial designed to evaluate the clinical benefit of neoadjuvant docetaxel in combination with ADT for patients with high-risk clinically localized prostate cancer. Residual tumor cells in the prostate after neoadjuvant therapy may possess characteristics representing intrinsic resistance or the early emergence of resistance pathways. Though these resistant cells may likely require additional ‘hits’ to trigger clinical progression, we hypothesized that resistant pathways observed in metastatic CRPC may be detected in a subset of patients treated on CALGB 90203.

Obtaining sufficient quality and quantity of nucleic acids from archival specimens including diagnostic needle biopsy tissue and post-treatment prostatectomy tissue required to test this hypothesis is a considerable challenge. In this study, we established and validated protocols amenable to formalin-fixed tissues to demonstrate the feasibility of obtaining robust targeted exome and transcript data. Based on prior studies, we know that AR signalling is re-activated in the majority of CRPC which can occur through activating mutations or amplifications involving the AR gene, expression of AR splice variants (eg., AR-V7), intra-tumoral steroidogenesis, or other mechanisms (34,35). AR mutations and copy number gains, present in 60% of metastatic CRPC tumors, are not observed in untreated primary tumors. To our knowledge, no prior studies have examined how rapidly AR genomic alterations emerge during initial ADT and which primary lesions are most likely to develop AR alterations. The notable paucity of AR mutations or amplifications by deep sequencing of the AR gene in our study suggests that these are typically later events. Although AR-V7 expression increased along with wild type AR gene expression, the AR-V7/AR-WT ratio remained unchanged in treated specimens. Despite no obvious changes in AR itself, the degree of AR signalling activity and the depth of AR target suppression did vary between patients and were dynamic in patient-matched specimens before and after neoadjuvant therapy, consistent with that observed in prior early phase neoadjuvant ADT trials (16,17,19). Overall these data suggest that AR signalling is relevant at early time-points and potentially driving response and resistance to therapy, yet is not likely driven through the common mechanisms associated with later AR reactivation in CRPC.

Genomic and transcriptomic alterations in several other signalling pathways, including PI3K/AKT, WNT, and DNA repair are enriched in advanced prostate cancer, and in some cases are capable of driving CRPC progression (8,36). Since primary treatment-naive prostate cancer is heterogeneous and often multi-focal within a single patient(37-40), whenever feasible, we analyzed spatially distinct macrodissected tumors. As expected, we observed commonly reported prostate cancer genomic alterations (eg., SPOP math domain missense mutations, PTEN loss) as well as alterations associated with poor prognosis disease (eg., loss of function alterations involving TP53 and RB1), consistent with the high-risk cases enrolled in this trial. Three of the 52 patients harboured pathogenic germline DNA repair gene mutations, recently reported in up to 12% of metastatic prostate cancer (41), but with an unclear etiologic role in high-risk disease. Targeted sequencing indicated an overall reduction in tumor cellularity
induced by treatment, but with marked variability between patients. Low tumor cellularity at prostatectomy, despite an initial diagnosis of high volume high grade disease (by biopsy), may be an indicator of therapy response and should be explored in the final trial analyses. In some patients, high volume residual disease remained and was marked by high allele frequency mutations and distinct transcript profiles, suggesting enduring fitness of certain clones. It is plausible that neo-adjuvant therapy shapes the clonal architecture and this hypothesis can be explored with more CALGB 90203 cases and clinical follow-up. Further, the incidence and clinical impact of germline alterations involving DNA repair genes in this expanded high-risk clinically localized prostate cancer cohort will be elucidated.

In a subset of CRPC, loss of AR signalling dependence can occur and this is often associated with lineage plasticity, activation of distinct transcriptional and epigenetic neuroendocrine-lineage pathways, and/or genomic alterations involving RB1, TP53 and MYCN (10,29-31,42). While most studies looking at lineage plasticity and the neuroendocrine phenotype have focused on CRPC, in this study we identified cases in which changes in these pathways could be detected early in a subset of patients treated with neoadjuvant therapy. This may represent an early adaptive response or possibly early acquired-resistance. Evaluation of pre-treatment biopsies was needed to elucidate pre-existing changes from those that were treatment induced. There is likely variability of AR dependence in individual patients with high-risk localized prostate adenocarcinoma and early neuroendocrine reprogramming and epithelial plasticity may start to emerge in outliers even in the context of short-term therapy, a framework further supported by our previously reported dynamic patient-derived xenograft (PDX) model (27,43) derived from a similar AR positive, PSA positive untreated tumor from a high-risk clinically localized prostate cancer patient. The tumor, which was TP53 and RB1 null, developed neuroendocrine prostate cancer upon PDX castration with retention of genomic alterations from the parent tumor and acquisition of neuroendocrine histologic and gene expression changes including suppressed AR signaling. These CALGB 90203 observations have implications in understanding the acute tumor response to therapy, possibly representing a reversible or ‘plastic’ state, and may inform the development of future prognostic biomarkers.

Preclinical data combined with clinical observations have provided insights into docetaxel resistance and have functionally linked stress chaperones (11-13) as well as the cyclin/CDK/RB pathway and ERG rearrangement status to taxane resistance (14,15). Clinical trial data suggest that the AR-V7 variant is not associated with taxane resistance (44,45). The extended datasets that will be generated by CALGB 90203 coupled with clinical features and outcomes and compared with ongoing contemporary neoadjuvant ADT studies may help dissect the relative contributions of AR versus taxane resistance in the hormone naive clinical setting.

In this proof of principle study, we demonstrate the feasibility of obtaining robust and reliable genomic and transcript data with small amounts of neoadjuvant treated, archival tissue. We were able to detect, quantify, and verify tumor mutations, AR signalling patterns, AR splice variant expression, and fusion transcripts. Limitations include the minimal tumor present in pre-treatment needle biopsies and post-treated tumors and the methods used for storing archival tissue specimens (as formalin fixed rather than banked fresh/frozen), both of which preclude
more extensive molecular analysis. Therefore, integrative analyses are limited to a select panel of genes, and the discovery of new resistance genes or other pathways including the analysis of intratumoral androgens was not picked up through these methods. Further, other readouts of biologic pathway activation including proteomic, metabolomic and immunologic changes could not be identified using this approach. Primary prostate cancers are commonly multi-focal, and paired analyses comparing biopsies and prostate RP specimens may also be confounded by intra-patient heterogeneity. Ongoing efforts are focused on extending the analysis to additional patients in the CALGB 90203 cohort, inclusion of additional genes to the panels, assessment of spatially distinct tumors when feasible, analysis for frequency of germline DNA repair alterations in this high-risk prostate cancer cohort, and clinical correlation with clinical variables and the pre-specified endpoints of the trial.

**Figure Legends**

**Figure 1. Study overview.** A) Schematic illustrating clinical trial design and the nature and number of tissue specimens available for downstream analysis. B) Example photomicrographs of H&E stained radical prostatectomy specimens from the treatment arm demonstrating differing degrees of treatment score. See also Supplementary Figure 1. C) Schematic showing the breakdown of genomic and transcriptomic profiling techniques applied to each patient in the cohort. The effect of treatment on serum prostate specific antigen (PSA) levels is also provided.

**Figure 2. Somatic mutations detected in diagnostic biopsy cores and matched radical prostatectomy specimens.** The bar plot shows minimum tumor cellularity predicted from targeted DNA sequencing of 72 prostate cancer driver genes in biopsy (upper) and radical prostatectomy (RP; lower). For some specimens, no somatic mutations were detected within the target regions meaning that tumor cellularity was unknown (indicated by lack of yellow or green bars). Mutations in frequently altered genes are provided in an oncoprint (also see Supplementary Figure 3). The bottom plot is a heatmap highlighting persistent AR expression in post-treated specimens (seven patients did not have expression data available and are indicated with filled gray boxes).

**Figure 3. Differentially expressed genes between treated and untreated patient subgroups.** Differentially expressed genes (n=57) were calculated based on a t test with corrected p-values < 0.15 (Benjamini-Hochberg method). Red= high expression; blue= low expression. Gene categories are highlighted and AR signalling score (red= high, blue= low), TMPRSS2-ERG fusion transcript (green= negative, orange= positive), and treatment status (yellow= untreated or blue= treated arm) are annotated.

**Figure 4. Differences in the expression of the androgen receptor (AR), AR-variant 7, and PSA between treated and untreated subgroups.** A) AR and AR-V7 gene expression in untreated and treated specimens. B) Change of tissue PSA expression (KLK3) versus change in serum PSA levels in untreated and treated patients. R= Pearson’s correlation.

**Table 1:** Baseline clinical characteristics. DOC = docetaxel; ADT = androgen deprivation therapy; IQR = inter-quartile range. Also see Supplementary Table 1.
References


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FIGURE 1

A

**Diagnostic biopsy**
Clinically localized high-risk prostate cancer (n=52)
Randomized 1:1

**Arm A**
neo-adjuvant docetaxel & ADT

**Arm B**
NO neo-adjuvant therapy

**Radical prostatectomy**

1mm FFPE tumor punch cores

**DNA**
(2-4 punches)

mRNA
(4-6 punches)

Pathology review and tissue macrodissection

**mRNA**

**DNA**

**mRNA**

**DNA**

Targeted DNA-seq possible in 44 patients
31 pairs

Targeted mRNA expression profiling in 34 patients

Microarray profiling

Targeted mRNA expression profiling in 45 patients
45 pairs

Targeted DNA-seq possible in 46 patients

Exome-seq (n=20)

Exome-seq (n=18)

RNA-seq (n=7)

Microarray profiling

B

Mild treatment effect

Moderate treatment effect

Strong treatment effect

20X

C

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<tr>
<td>PSA pre-surgery</td>
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**Biopsy tumor**
Targeted DNA-seq - Nanostring mRNA
Targeted DNA-seq (tumor & benign)
Nanostring mRNA (tumor & benign)
Exome-seq (FFP tumor & benign)
RNA-seq (FF tumor)
FIGURE 3

Color Key and Density Plot

- Treated
- Untreated

Row Z-Score

AR signaling score

TMPRSS2-ERG

Treatment

VIP
SST (somatostatin)
Sox2
HES1
CDKN1B
ATM
FBXW7
REST
PI3K2B
CCND1
PTHLH
HOXA11
ESR2
DLL1
TGFBR2
MET
FGFR2
NR3C2 (MCR)
NR3C1 (GCR)
ZEB2
NOTCH1
PGR (NR3C3)
CCND2
CD200
ENO2 (NSE)
DTX3
NOV3
NAN1
vim
Fscn1
Serpin2
DLL4
HEY1
PTEN
MAPRE2
MAN1A1
KDR (VEGFR2)
JAKMIP2
CHD1
SNAI1
CDKN1A
IL6
PCSK2
ESR1
HELLS
DNMT1
BRCA2
AR-RV7
AR
PEG10
CD56 (NCAM)
WNT5A
DDC
CHGA
ASCL1
NPY
KLK2
ERG

Androgen Regulated
Cell Cycle
NEPC Regulated
Other

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**FIGURE 4**

A

**AR**

- Untreated Treated
- Log2(normalized counts)

**ARv7**

- Untreated Treated
- Log2(normalized counts)

B

**Untreated**

- PSA log2 (fold change)
- KLK3 log2 (fold change)
- R = 0.23

**Treated**

- PSA log2 (fold change)
- KLK3 log2 (fold change)
- R = 0.36

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Clinical Cancer Research

Impact of therapy on genomics and transcriptomics in high-risk prostate cancer treated with neoadjuvant docetaxel and androgen deprivation therapy


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