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Modeling A Lethal Prostate Cancer Variant with Small Cell Carcinoma Features

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Abstract

Purpose—Small-cell prostate carcinoma (SCPC) morphology predicts for a distinct clinical behavior, resistance to androgen ablation, and frequent but short responses to chemotherapy. We sought to develop model systems that reflect human SCPC and can improve our understanding of its biology.

Experimental Design—We developed a set of CRPC xenografts and examined their fidelity to their human tumors of origin. We compared the expression and genomic profiles of SCPC and large cell neuroendocrine carcinoma (LCNEC) xenografts to those of typical prostate adenocarcinoma xenografts. Results were validated immunohistochemically in a panel of 60 human tumors.

Results—The reported SCPC and LCNEC xenografts retain high fidelity to their human tumors of origin and are characterized by a marked upregulation of *UBE2C* and other mitotic genes in the absence of *AR*, retinoblastoma (*RBI*) and cyclin D1 (*CCND1*) expression. We confirmed these findings in a panel of CRPC patients' samples. In addition, array comparative genomic hybridization of the xenografts showed that the SCPC/LCNEC tumors display more copy number variations than the adenocarcinoma counterparts. Amplification of the *UBE2C* locus and microdeletions of *RBI* were present in a subset, but none displayed *AR* nor *CCND1* deletions. The *AR*, *RBI*, and *CCND1* promoters showed no CpG methylation in the SCPC xenografts.

Conclusion—Modeling human prostate carcinoma with xenografts allows in-depth and detailed studies of its underlying biology. The detailed clinical annotation of the donor tumors enables associations of anticipated relevance to be made. Futures studies in the xenografts will address the functional significance of the findings.

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Keywords

castration resistance prostate cancer; small cell neuroendocrine carcinoma; retinoblastoma; UBE2C; cyclin D1

Introduction

Small cell carcinoma morphology is often found on repeat biopsies during castrate-resistant prostate carcinoma (CRPC) progression(1-3). Its presence predicts for a course characterized by frequent visceral metastases, predominantly lytic bone lesions, and large prostatic or nodal masses, which is distinct from that of the typical bone-homing, hormone-responsive prostate carcinoma (PCa). Small-cell prostate carcinomas (SCPCs) do not express androgen receptor (AR) and thus do not respond to androgen ablation. They frequently respond to chemotherapy but responses are short and median survival stands at approximately 1 year(1-3). An understanding of the biology underlying this lethal subset of PCa is urgently needed, to identify an objective molecular signature and specific treatments that improve the outcome of afflicted men.

We previously characterized 8 SCPC and large-cell neuroendocrine (NE) carcinoma (LCNEC) xenograft tumor lines derived from a single patient's tumor(4). LCNEC is a rare morphologic variant belonging to the spectrum of poorly differentiated NEPCa(5-7). Expression analysis of various molecular markers revealed gradual changes in the donor tumor's different morphologic components, suggesting that SCPC and LCNEC are part of a biologic continuum. Although limited, because they were derived from a single patient's tissue, those initial findings provided a foundation to study the biology of poorly differentiated NEPCa(4). To obtain further insight into this biology we developed and characterized additional high-fidelity human CRPC tumors with and without SCPC/LCNEC morphology.

Loss of retinoblastoma (RB) function and upregulation of mitotic genes, including the UBE2C-anaphase promoting complex (APC) pathway, have recently been implicated in the progression to castration-resistance in PCa mediated by AR activity(8, 9). In the study reported here, we show that mitotic genes (including UBE2C) are markedly upregulated in SCPC and LCNEC xenografts, in the absence of AR, RB and cyclin D1 expression. Moreover, we demonstrate that these findings are mirrored in human CRPC tumors and thus show that these novel xenograft models share the biologic properties of the human disease and are suitable for the identification of candidate pathways that may drive SCPC/LCNEC biology. The parallel understanding of the functional relevance in murine systems and clinical cohorts should accelerate the development of candidate therapies for this lethal PCa variant.

Materials and Methods

Primary Donor Tumors and Xenografts

The xenograft models MDA PCa 79, 117, 130, 170, 180, 144, 146, and 155 were derived from the primary CRPCs (donor tumors) acquired during palliative surgical resections at The University of Texas MD Anderson Cancer Center from 8 patients who provided written informed consent (IC) on an IRB-approved protocol. Donor patients of xenografts 144 and 155 were enrolled in a clinical trial designed to test the chemotherapy sensitivity of CRPC with SCPC or with clinical features associated with SCPC, a clinical syndrome termed "anaplastic" PCa.(10)

Xenograft development is described in Supplementary Data. All animal experiments were approved by our Institutional Animal Care and Use Committee and conducted following accepted standards of care. In the case of MDA PCa 117, 170, 180, 144, 146, and 155, different tissue fragments were implanted in several different mice, resulting in the development of multiple sublines. Tumor samples from locations immediately adjacent to the samples used for implantation were processed for histopathologic and immunohistochemical (IHC) analyses.

Tissue sections from paraffin blocks of all xenograft lines and sublines were reviewed and representative blocks selected to construct a tissue microarray (TMA) containing 0.6-mm-diameter cores. Thirty-eight blocks (median, 2; range, 1–3 blocks/xenograft) were sampled, and 117 cores (median, 6; range, 3–9 cores/xenograft) were included in the “Xenograft TMA.”

Gene-expression Array

Gene expression was profiled as previously described (4). Briefly, total RNA was extracted using an RNeasy mini kit (Qiagen, Inc. Valencia, CA) from multiple tissue fragments of MDA PCa 79 ($n = 3$), 117-9 ($n = 3$), 130 ($n = 2$), 144-4 ($n = 5$), 144-13 ($n = 5$), 146-10 ($n = 3$), 155-2 ($n = 1$), and 155-12 ($n = 1$) tumors and submitted to the MD Anderson Genomics Core Facility for conversion to cDNA, labeling and hybridization to a U133A 2.0 Plus array (Affymetrix, Inc. Santa Clara, CA). The methods of data analysis and quantitative reverse-transcription PCR validation with SYBR Green are provided in Supplementary Data and Supplementary Table 1.

Human CRPC Sample Selection

We searched the tissue bank files of the MD Anderson Department of Pathology to identify surgically excised locally advanced CRPC samples from patients providing written IC allowing use of their tissue for research ($n=46$). We also included biopsy specimens of SCPCs with sufficient tissue for IHC analysis ($n=14$). In total, we studied 68 “clinical CRPC samples” obtained from 60 patients: 24 SCPCs/LCNECs and 44 prostatic adenocarcinomas (including the SCPC/LCNEC and adenocarcinoma components of 8 mixed cases) (Supplementary Data). Their clinicopathological history was extracted retrospectively from their medical records under an IRB-approved protocol.

IHC analyses

Tissue sections (4 μ m) from the xenograft's donor tumors, TMAs, and biopsy specimens were subjected to IHC analyses, using an Autostainer Plus (Dako North America, Inc. Carpinteria, CA). Details of IHC procedures are presented in Supplementary Data and Supplementary Table 2. Chromogranin A and synaptophysin stains were considered positive if >5% of cells stained positively. For all other markers, the percentage of positive cells was calculated as the number of positively stained epithelial cells divided by the total number of epithelial cells, at X200 magnification. Owing to the limited tissue, biopsy specimens were only stained with anti-UBE2C, -cyclin D1, and -RB (Calbiochem) antibodies.

Array comparative genomic hybridization (aCGH) analysis

Genomic DNA was extracted from fresh-frozen xenograft specimens (1 each from MDA PCa 144-13, 144-4, 146-10, 155-2, 170-4, and 180-30) and from peripheral blood mononuclear cells (PBMC) from healthy male volunteers providing written IC by using standard proteinase K and phenol–chloroform methods. After quantification by A260 on a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific NanoDrop Products), 5 μ g of genomic DNA per sample were submitted to our Genomics Core Facility, where tumor

DNA was labeled with Cy3 and PBMC DNA with Cy5 dye, and cohybridized on a Human Genome CGH Microarray 244A (Agilent Technologies, Inc. Santa Clara CA). Quality control included correlation between reference channels, data distribution plots, and principal component analysis.

Quantitative Reverse-Transcription PCR, Flow Cytometry, Western Blot, Cell Line Culture and Pyrosequencing are described in Supplementary Data

Statistical analyses

Continuous data were summarized with descriptive statistics (i.e., mean with SD), and categorical data, with the use of contingency tables. Two-sample *t*-testing assessed for mean between-group differences in continuous variables, and chi-square testing, for differences in categorical-variable distributions. Pearson's rank correlation coefficient testing was used to assess correlation in expression between biomarkers. Cohen's kappa coefficient test was used to assess agreement amongst the RB antibodies. All *P*-value determinations were 2 sided at a 0.05 significance level. SPSS for Windows release 16.0 software was used for the analyses.

Gene-expression array analysis was previously described(4). Briefly, robust multichip analysis was used for data normalization and quantification. One-way ANOVA was applied to identify differently expressed genes. The beta uniform mixture model (11), false discovery rate (FDR), and Tukey's honestly significant difference were combined to adjust for multiple testing.

Analysis of aCGH was done using genomic segmentation, as implemented in the Partek Genomics Suite Version 6.5 (Partek Inc., St Luis, MO), and performed using the following parameters: 20 minimum genomic markers, 2-sided *t* test *P* value threshold 0.0001, and signal-to-noise ratio >0.4. To study the linear relationship between copy-number variations (CNVs) and gene expression data, we first categorized CNVs in several levels. Then corresponding genes were extracted for each CNV, their average expression values were calculated, and linear regression was used to assess the relationship between CNV levels and gene-expression data.

Results

CRPC xenografts retain fidelity to the human tissues of origin

The clinicopathological features of the 8 xenograft donor patients are summarized in Table 1. The MDA PCa 144 xenografts have been described in detail (4), but the rest are described here for the first time. MDA PCa 170, 180, 146, and 155 tumors produced several xenograft sublines.

The SCPC/LCNEC xenografts (MDA PCa 144, 146, and 155) were AR⁻ and PSA⁻, expressed ki67 highly, and stained positively for chromogranin and synaptophysin. Additionally, all SCPC/LCNEC xenografts displayed intense nuclear staining for p53, as did their respective donor tumors (data not shown). MDA PCa 79, 117, 130, 170, and 180 xenografts had the morphologic features of conventional adenocarcinoma and expressed high levels of AR but low levels of ki67. The MDA PCa 117 xenograft was chromogranin A⁺ and synaptophysin⁺, and the MDA PCa 180 was synaptophysin⁺, as were their respective donor tumors. Table 2 and Figure 1A show that the xenografts' (and their sublines') histopathologic and IHC features reflect those of their donor tumors. Supplementary Figure 1A shows the growth rate of 144-13, 146-10 155-2, 170-4 and 180-30 xenografts.

Gene-expression profiling of SCPC/LCNEC xenografts reveals upregulation of mitotic genes

Unsupervised hierarchical clustering of the raw expression profiles obtained from the MDA PCa 79, 117-9, 130, 144-13, 144-4, 146-10, 155-2, and 155-12 xenografts showed that replicates from the same xenograft clustered together, reflecting biologic identity, and that the xenografts were classified according to their morphology and AR expression (Supplementary Figure 1B).

SCPC/LCNEC xenografts were collectively compared against the adenocarcinoma xenografts. We identified 140 (0.3%) probes expressed differently (FDR, 0.05), corresponding to 104 unique RefSeq Transcript IDs (Supplementary Table 3). Gene Ontology analysis showed enrichment in mitosis-related biologic-process subtrees, including “cell-cycle phase” (adjP = $2.68e^{-8}$), “mitotic cell cycle” (adjP = $2.68e^{-8}$), and “cell division” (adjP = $2.68e^{-8}$), among the 104 genes expressed differently between the groups (Supplementary Figure 1C). Genes in these subtrees included *CDC25C* (cell division cycle 25C), *ANLN* (actin-binding protein anillin), *AURKA* (aurora kinase A), *HELLS* (helicase, lymphoid-specific), *UBE2C* (ubiquitin-conjugating enzyme E2C), *PTTG1* (pituitary tumor-transforming gene 1), *KPNA2* (importin subunit alpha-2), *TACC3* (transforming acidic coiled-coil-containing protein 3), *PDCD6IP* (programmed cell death 6-interacting protein), *HIST1H4C* (histone H4), and *PCNA* (proliferating-cell nuclear antigen).

Wang et al. (9) recently reported that in a CRPC model, AR upregulated a set of M-phase genes, including *UBE2C* (an anaphase promoting complex [APC] specific ubiquitin-conjugating enzyme) to promote tumor growth. However, a thorough examination of AR expression in SCPC/LCNEC xenografts confirmed their lack of AR mRNA (including exon-2 splice variant) and protein (Figure 1B, C). We then confirmed the markedly increased expression of *UBE2C* in the AR-negative SCPC/LCNEC xenografts relative to that in the AR-positive adenocarcinoma xenografts by qRT-PCR ($P = 0.003$) (Figure 1B) and IHC ($P = 0.002$) (Table 2, Figure 1C) analyses. qRT-PCR also revealed higher expression of other APC members, such as *Cdc20* ($P = 0.04$) and *FZR1/CDH1* ($P = 0.064$) in the SCPC/LCNEC xenografts, further supporting the notion that APC activity is deregulated in this subset of PCa (Supplementary Figure 2).

SCPC/LCNEC xenografts lack cyclin D1 and RB expression

Visual analysis of H&E stains revealed higher number of mitotic figures and Ki67 expression in the SCPC/LCNEC xenografts relative to the adenocarcinoma ($p < 0.001$) (Supplementary Figure 3A,B) which might suggest that the M-phase gene overexpression is a mere reflection of the increased proliferation of SCPC/LCNEC tumors. However, except for 144-13 (which contains large amounts of necrosis) the growth rate of the SCPC/LCNEC xenografts was not significantly different from that of the adenocarcinoma xenografts (Supplementary Figure 1A). It has been shown that not all cells containing the Ki-67 antigen are actively proliferating cells and that tumor cells can remain positive for Ki-67 even when arrested in G1/S or G2/M (12). Indeed, flow cytometry analysis showed that the SCPC 146-10 xenograft contained a higher proportion of cells in G2/M phase compared to the adenocarcinoma 180-30 xenograft, while the proportion of cells in S phase was similar in both (Supplementary Figure 3C) suggesting that the overexpression in *UBE2C* and other mitotic genes could reflect an accumulation of cells in mitosis rather than increased proliferation.

We searched for additional cell cycle-gene-expression abnormalities to support this concept. The absence of cyclin D1 mRNA in the SCPC/LCNEC xenografts was striking because cyclin D1 is frequently overexpressed in cancers(13) and its increased expression has been

associated with a worse prognosis in PCa(14). Indeed, primers mapping to the 5' end of cyclin D1 cDNA, encompassing both cyclin D1a and D1b transcripts(15), confirmed that cyclin D1 mRNA levels were markedly lower in the SCPC/LCNEC than in the adenocarcinoma xenografts ($P = 0.053$) and IHC showed this was also true for protein levels ($P = 0.002$)(Figure 1B-C, Table 2).

Since cyclin D1 loss might bypass the need for RB protein, and loss of RB function is virtually universal in small-cell lung carcinoma, a tumor with many clinical and pathologic similarities to SCPC(16, 17), we measured RB transcript levels encompassing exons 2/3 of the gene. We found lower (but present) levels of RB mRNA in the SCPC/LCNEC (mean, 23.6; SD, 21.3) than in the adenocarcinoma xenografts (mean, 45.1; SD, 3.6), but the difference was not statistically significant ($P = 0.271$) (Figure 1B). IHC analysis, however, showed complete absence of RB protein expression in the SCPC/LCNEC xenografts but abundantly positive staining in the adenocarcinoma xenografts ($P = 0.003$) (Figure 1C, Table 2). To strengthen these observations we used three different antibodies against RB (Supplementary Table 2) and obtained highly concordant results (data not shown) as determined by Pearson's correlation coefficient testing for continuous variables ($r > 0.89$, $P < 0.001$ for all correlations) and the kappa statistic for variables dichotomized by 10% ($\kappa > 0.86$, $P < 0.001$ for all correlations).

SCPC/LCNEC xenografts display Copy Number Variations (CNVs) and *RB1* microdeletions but no *AR* or *CCND1* deletions

Because both *UBE2C* overexpression and RB loss have been implicated in tumor chromosomal instability(18, 19), we performed aCGH analysis to compare CNVs between the SCPC/LCNEC and adenocarcinoma xenografts. Principal component analysis of the samples showed that MDA PCa 170-4 and 180-30 xenografts clustered separately from the rest, and within the SCPC/LCNEC group, the two MDA PCa 144 samples also clustered together (Figure 2A). A histogram of the copy number from all segments revealed separation of peaks at 1.6 and 2.4. Using those as cutoffs, we identified 446 amplifications, 431 deletions, and 435 unchanged segments. Indeed, the SCPC/LCNEC MDA PCa 144-4, 144-13, 146-10, and 155-2 xenografts displayed more amplifications (87 ± 41.5) and deletions (69.5 ± 18.2) than did the adenocarcinoma MDA PCa 170-4 and 180-30 xenografts (15 ± 11.3 and 28 ± 5.7 , $P = 0.03531$ and 0.01524 , respectively)(Figure 2B).

In SCPC/LCNEC xenografts, 8 regions were commonly amplified in chromosomes 1, 5, 8, and 21, and 16 regions were commonly deleted in chromosomes 10, 12, 13, 15, 17, and X (Supplementary Figure 4A). CNVs were categorized into levels according to copy number: -2 (mean, <1), -1 (mean, 1 <1.6), 0 (mean, 1.6 <2.4), 1 (mean, 2.4 <3), 2 (mean, 3 <4), and 3 (mean, 4). Linear regression analysis showed significant linear relationships between the categorized CNVs and gene-expression values for the MDA PCa 144-4, 144-13, 146-10, and 155-2 xenografts in the array ($P < 2.2e^{-16}$). Of note, given the small number of samples, we applied strict criteria to call a CNV, ensuring specificity. Thus, the number of CNVs in the adenocarcinoma samples is relatively small compared to previous aCGH reports in CRPC.(20)(21) Although looser criteria might increase the number of CNVs in each group, the conclusion that the number of CNVs in SCPC/LCNEC is greater than that in the adenocarcinomas would be unlikely to change.

We asked whether detected CNVs might explain the differences in gene expression between the SCPC/LCNEC and the adenocarcinoma xenografts. Overexpression of *UBE2C* has been attributed to amplification of the *UBE2C* locus at 20q13.1 in various tumors(22). Xenograft MDA PCa 155-2 displayed a log ratio of 0.63, corresponding to a copy number of 3.1 at the 20q13.12 segment containing the *UBE2C* gene. No other xenografts showed amplifications in this region (Figure 2C). Xenograft MDA PCa 146-10 displayed a log ratio of 0.64,

corresponding to a copy number of 3.12 at the 11q13.2 segment containing the *CCND1* gene despite the absence of cyclin D1 mRNA in this tumor. No other xenografts showed CNVs in this region (Figure 2C). Xenografts MDA PCa 155-2, 170-4 and 180-30 showed log ratios of 0.4, 5.2 and 0.5, corresponding to copy numbers of 2.64, 74.54 and 2.78, respectively, at the Xq12 segment containing the *AR* gene, although *AR* mRNA is absent in MDA PCa 155-2 (Figure 2C). Finally, diverse microdeletions were observed at the 13q14 segments containing *RB1* in the SCPC/LCNEC but not the adenocarcinoma xenografts (Figure 2C). Additional studies, with greater numbers of samples, are required to confirm the presence or absence of CNVs affecting the *UBE2C*, *CCND1*, *RB* and *AR* genes in SCPC tumors.

SCPC/LCNEC xenografts do not express full-length RB1 protein

Given the aCGH results, we designed additional qRT-PCR primers to map the *RB1* gene's exons 18/19, 21/22, and 26/27 (Figure 3A). As shown in Figure 3B, MDA PCa 144-13, which displayed a 3' microdeletion of the *RB1* gene on aCGH, expressed mRNA encompassing exons 2/3 but none encompassing the distal exons. MDA PCa 155-2 expressed mRNA for all but the regions encompassing exons 18/19, whereas MDA PCa 146-10 expressed mRNA for all of the mapped exons. In contrast, the adenocarcinoma xenografts MDA PCa 170-4 and 180-30 expressed all of the mRNA regions tested. These observations were further supported by RT-PCR of exons 17–27 (Figure 3C). Western blot analysis confirmed that the AR⁻ SCPC/LCNEC MDA PCa 144-13, 146-10 and 155-2 xenografts do not express full-length RB protein, whereas the AR⁺ CRPC xenografts with adenocarcinoma morphology (MDA PCa 170-4 and 180-3) do (Figure 3C).

AR, cyclin D1, and RB promoter regions are not methylated in SCPC/LCNEC xenografts

Because *AR* promoter methylation has been described as a mechanism underlying *AR* loss in AR⁻ cell lines(23) and both the *RB1* and *CCND1* promoters contain CpG islands(24,25), we asked whether promoter methylation could explain the *AR*, *RB*, and cyclin D1 silencing found in the SCPC/LCNEC xenografts.

Consistent with previous reports, the *AR* promoter region was aberrantly hypermethylated by pyrosequencing analysis in the AR⁻ PCa cell lines PC-3 and DU145 but not in the SCPC/LCNEC xenografts and neither were the *RB1* or *CCND1* promoters (Supplementary Figure 4B).

IHC analysis confirmed the UBE2C^{high}, AR⁻, cyclin D1⁻, RB⁻ signature in human SCPC tissues

To validate our observations we conducted IHC analysis of a 68-sample panel of “Clinical CRPC Samples” described above. The clinicopathologic characteristics of the 60 patients from whom they were obtained are described in Supplementary Table 4. Among the anti-RB antibodies used, the Calbiochem antibody was further analyzed because of its more-intense nuclear staining and less-intense cytoplasmic background staining.

Indeed *UBE2C* expression was markedly increased while cyclin D1 and *RB* levels were markedly decreased in the clinical CRPC samples with SCPC/LCNEC morphology relative to those with adenocarcinoma morphology ($P < 0.001$ for all comparisons). This was true in SCPC samples obtained from primary SCPC (n=3), SCPC samples obtained prior to starting androgen deprivation therapy (n=2) and SCPC samples obtained prior to start of chemotherapy (n=9), suggesting that these observations reflect the biology underlying SCPC and are not a consequence of prior treatment. Of note, the mixed adenocarcinomas had lower levels of cyclin D1 and *RB* than did the pure adenocarcinomas ($P < 0.001$ for both comparisons)(Figure 4A).

Using 10% positive cells as a cut-off, 23/24 (96%) SCPC/LCNEC clinical CRPC samples had high UBE2C expression, whereas only 12/36 (33%) pure adenocarcinomas and 2/8 (25%) mixed adenocarcinomas showed high levels of UBE2C (Figure 4A). All but 1 (96%) of the SCPC/LCNEC clinical CRPC samples were RB⁻/cyclin D1⁻ while 34/44 (77%) adenocarcinomas were RB⁺/cyclin D1⁺. Interestingly, three (7%) of the adenocarcinomas were RB⁺/cyclin D1⁻, but the intensity of RB expression in these cases was only 1+, whereas all RB⁺/cyclin D1⁺ cases stained intensely (2+ and 3+) for RB. Also, it is noteworthy that 5/7 (16%) of the RB⁻/cyclin D1⁻ adenocarcinomas were mixed with SCPC or LCNEC (Figure 4B). PSA, ki67, chromogranin and synaptophysin staining results in the clinical CRPC samples are described in Supplementary Data.

Discussion

The models described here were derived from patients with a well defined clinical history of anaplastic PCa (10) selected in a non-random way and demonstrated high degree of fidelity to their donor tumor. The results of the molecular studies were robustly validated in a cohort of patients with CRPC that shared the phenotypic features of the xenografts and their donor tumors. These findings support the notion that these xenografts can be used to model SCPC in order to fill the knowledge gap in their biology, predict for its emergence in CRPC and develop specific therapies to improve the outcome of men afflicted with this lethal PCa variant. The cellular program that drives the growth of the AR⁻ SCPC/LCNECs variants is a mechanism of escape from the AR-ablating treatments currently at the forefront of PCa therapy. Moreover, the pathways implicated in the pathogenesis of this subset may also contribute to castration resistance in PCa with non-variant morphology. Using xenograft models as discovery platforms, we found high expression of M-phase genes, including UBE2C, coupled with RB and cyclin D1 loss in SCPC/LCNEC, despite the absence of AR expression. The robust validation of these findings in patient CRPC samples show that xenograft models can contribute significantly to the understanding of human PCa biology and may serve as tools for the efficient prioritization of candidate therapies.

The origin of SCPC has long been debated(26-28). The frequent coexistence of SCPC and adenocarcinoma, the presence of transition areas between the 2 morphologic types in mixed tumors(3, 4), and the *TMPRSS2:ERG* fusions and *TP53* mutations that are shared by the components of mixed tumors(29, 30) all support the concept of a common derivation, either through transdifferentiation of one cell type to the other or through a common stem-cell origin. All but one of the AR⁻ SCPC/LCNEC tumors we tested were also RB⁻ and cyclin D1⁻, whereas the “mixed adenocarcinomas” (the adenocarcinoma components of the mixed tumors), which were frequently RB⁻ and cyclin D1⁻, remained AR⁺ and had lower levels of UBE2C expression. These observations suggest a sequence of events in which loss of RB and/or cyclin D1 precede AR-loss and further deregulation of the mitotic apparatus. Understanding the sequence of events that drives the emergence of the SCPC phenotype during CRPC progression may lead to identification of prognostic markers for the early recognition of patients destined to develop this lethal variant of PCa.

UBE2C collaborates with the multiprotein complex APC in the degradation of mitotic proteins which is essential for cells to exit mitosis and progress through the cell cycle. Overexpression of UBE2C leads to uncontrolled APC activity and precocious degradation of cyclinB1 that leads to aneuploidy(18). UBE2C is overexpressed in human carcinomas of diverse anatomic origin(22) and UBE2C transgenic mice are prone to develop a broad spectrum of tumors supporting an oncogenic role for this protein (18). The mechanisms of UBE2C overexpression in cancer cells that do not express AR are poorly understood and likely to be complex. One recent report implicated Cdc20 in the activation of the UBE2C promoter.(31) Another demonstrated that phosphorylated coactivator mediator-1 (MED1)

and FoxA1 mediated long-range interactions between UBE2C enhancers and the UBE2C promoter, leading to UBE2C gene expression in PC-3 cells.(32) Our gene expression analysis showed increased Cdc20 (Supplementary Figure 2A) and FoxA1 (data not shown) expression in SCPC xenografts compared to adenocarcinoma, suggesting both mechanisms might be at play in this setting.

Interestingly, a recent report showed upregulation of aurora kinase A in human SCPC and provided evidence that its inhibition suppressed NEPCa growth (33). Aurora kinase A has also been shown to be upregulated in diverse human carcinomas and been ascribed an oncogenic role. We too observed upregulation of aurora kinase A (but not amplification), as well as numerous other mitotic genes, including aurora kinase B, polo-kinase 1 and FOXM1 (data not shown) in our SCPC models. Hence it appears that the mitotic apparatus is an important target in SCPC and fortunately, a number of mitotic kinase inhibitors are already in early clinical trials(34). However, the mitotic apparatus is complex, and selecting the optimal target will require an in-depth understanding of the hierarchy that drives the disease. Accurate preclinical models of the disease are needed to efficiently prioritize available drugs for clinical development. Despite previous publications advocating PC-3 as an accurate model of SCPC (35), we contend that significant differences exist between PC-3 and SCPC, including the presence of a wild-type *RB* (36) and the methylation of its AR promoter. The shared biologic properties between our xenografts and their donor tumors and the validation of our results in clinical SCPC/LCNEC samples provide evidence that they are valuable tools for modeling the disease and testing the efficacy of therapeutic strategies.

RB-loss has been implicated in the progression to CRPC via increased AR expression, mediated by E2F transcription factor 1(8). Indeed, the tumor-suppressor function of *RB* has been attributed mainly to its ability to inhibit E2F transcription factors and induce G₁ arrest. However, *RB* plays diverse cellular roles beyond that of a G₁ checkpoint (such as in chromosome condensation, centromeric function, and chromosome stability) and *RB*-loss has been directly implicated in the development of the chromosomal instability that characterizes poorly differentiated tumors (19). Hence, additional consequences of *RB*-loss that are not mediated by an increase in AR activity would be expected to emerge in the progression of CRPC.

The high *RB* expression we observed in the AR⁺ castrate-resistant adenocarcinomas samples is at odds with the recent report of *RB*-loss in CRPC(8). Two considerations might explain this discrepancy. First, Sharma et al(8) examined specimens from CRPC metastases, whereas our specimens were all derived from primary CRPC tumors. Perhaps *RB* status is discrepant between primary and metastatic sites. Second, bladder cancer studies have shown that intense *RB* protein expression can be attributed to protein hyperphosphorylation, which inactivates *RB*(37). Both low and high *RB* protein expression have been associated with nonfunctional *RB* protein in bladder carcinomas(38). In these studies *RB*-negative tumors were also cyclin D1⁻, whereas tumors that overexpressed *RB* had high cyclin D1. Perhaps in CRPC adenocarcinoma, aberrations of the G₁-S transition are mediated through cyclin D1 overexpression, leading to *RB* hyperphosphorylation, seen on IHC analysis as *RB* protein overexpression. Thus, different mechanisms of *RB* inactivation (loss of full-length protein expression versus hyperphosphorylation) might lead to different PCa phenotypes (small-cell carcinoma versus adenocarcinoma), as has been proposed in lung malignancies(39). Elucidation of these mechanisms gains further significance with the introduction of novel cyclin-dependent kinase (CDK) inhibitors, seeking to activate *RB*,(40) into clinical trials.

The loss of cyclin D1 expression in SCPC was puzzling because this protein is generally activated in proliferating cells and repressed in differentiated cells.(41) However, beyond its

ability to activate CDKs to initiate proliferation, cyclin D1 is a regulator of the activity of numerous transcription factors, including the AR(42) and exerts diverse effects depending on the cellular context. For example, Lehn et al(43) showed that cyclin D1 downregulation increased the migratory capacity of breast cancer cell lines and was associated with unfavorable prognostic features. Since cyclin D1 mRNA was undetectable but no loss or rearrangement of the cyclin D1 gene was noted, we propose that its loss is due to transcriptional repression. It has been shown that overexpression of E2F1 can inhibit cyclin D1 promoter activity.(44) In our study, cyclinD1 loss was strongly correlated with RB loss, and the gene expression arrays showed significant overexpression of E2F1 in the SCPC xenografts (data not shown). Thus we speculate that E2F1 repression might contribute to cyclin D1 silencing in the SCPC.

Similarly, although most studies have focused on the oncogenic effects of AR, substantive evidence also supports a tumor -suppressor role for it in PCa (45). For instance, in TRAMP mice, AR favored survival of differentiated tumor epithelium while suppressing proliferation of more-malignant basal intermediate cells(46). The mechanism by which AR expression is lost in SCPC is unknown and the subject of ongoing investigations in our laboratory.

SCPC often express NE markers such as chromogranin A and synaptophysin, and NE differentiation has been proposed to contribute to androgen-independence in PCa. However, the term “NE differentiation” in PCa encompasses a wide range of conditions. NE markers, such as chromogranin A and synaptophysin, are expressed in 10-100% of untreated tumors depending on the methodology used and the extent of cells that stain positively for these markers increases during castration.(47) Notably, the extent of NE marker expression considered to constitute NE differentiation in PCa has not been clearly defined. In our study, the MDA PCa 117 displayed conventional adenocarcinoma morphology and expressed NE markers. Within the clinical CRPC samples with conventional adenocarcinoma morphology, 21 expressed synaptophysin and 2 chromogranin (Supplementary Data). The expression of UBE2C, cyclin D1, Rb, AR and ki67 did not differ significantly in these cases compared to adenocarcinomas lacking NE marker expression. Therefore, the presence of NE differentiation in PCa, while possibly prognostic, is not predictive of a distinct clinical course nor a therapy response profile (48) and must not be confused with SCPC.

In summary, we have developed and characterized a panel of CRPC xenografts models that mirror the human tumors from which they were derived. Their study has allowed us to demonstrate that AR⁻ SCPC/LCNEC tumors display marked UBE2C overexpression, coupled with loss of RB and cyclin D1 expression, features which are likely to contribute to the pathogenesis and AR independence of this poorly understood lethal PCa variant. Hence, we provide evidence that xenograft models are representative of the human disease and can serve as valuable tools to define clinically relevant biology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Statement of Translational Relevance

Small cell prostate carcinoma (SCPC) is a lethal variant of prostate cancer (PCa). Androgen ablation therapy is ineffective, the frequently observed chemotherapy responses are transient and median survival stands at approximately one year. An improved understanding of the mechanisms of progression to SCPC is urgently needed and may have broader implications in PCa. We characterized unique human PCa xenografts from patients with advanced castration-resistant prostate carcinomas (CRPC) and found significant upregulation of mitotic phase genes, including UBE2C (an anaphase promoting complex specific E2 conjugating enzyme), and loss of retinoblastoma, cyclin D1 and androgen receptor expression in the SCPC xenografts when compared to xenografts with conventional adenocarcinoma morphology. The results were confirmed in the donor tumors and in a cohort of CRPC patients' samples. These novel models can be used as a platform to study the biology underlying SCPC and to prioritize candidate therapies and predictive markers for further study.

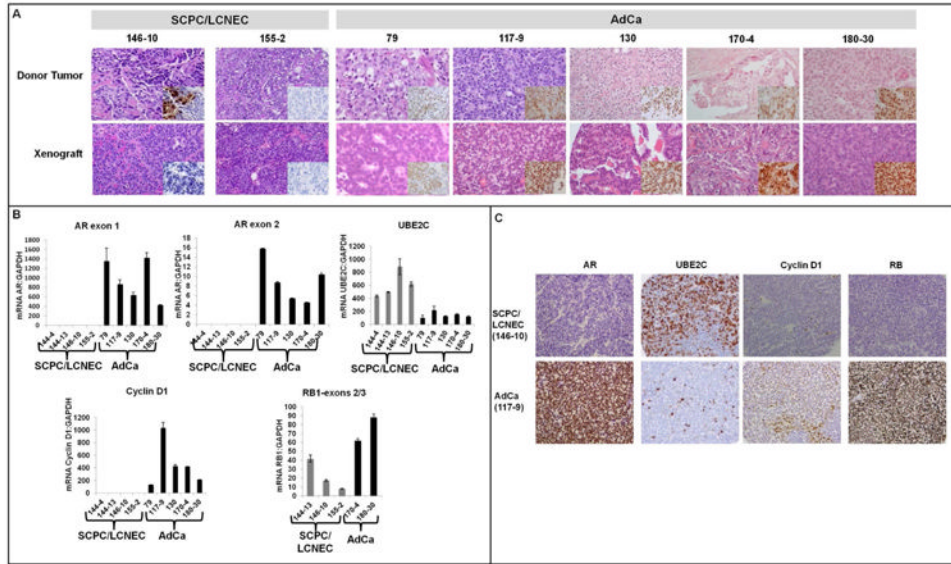


Figure 1. A, Representative images of the xenografts and their donor tumors, with hematoxylin and eosin staining; the insets show IHC staining for AR. Note that the MDA PCa 146-10 donor tumor was mixed, containing adenocarcinoma (AdCa) and SCPC components. The inset shows that the AdCa component was AR⁺, whereas the SCPC component was AR⁻. (Chr: chromogranin; Syn: synaptophysin. Original magnification of all images except 155-2, ×200, original magnification of 155-2 images, X100). B, mRNA levels of AR (exons 1 and 2), *UBE2C*, *cyclin D1*, and *RB1* exons 2/3 in SCPC/LCNEC (gray bars, MDA PCa 144-4, 144-13, 146-10, and 155-2) and AdCa xenografts (black bars, MDA PCa 79, 117-9, 130, 170-4, and 180-30). C, Representative images of AR, UBE2C, cyclin D1, and RB immunostaining in SCPC/LCNEC and AdCa xenografts. Original magnification, ×200.

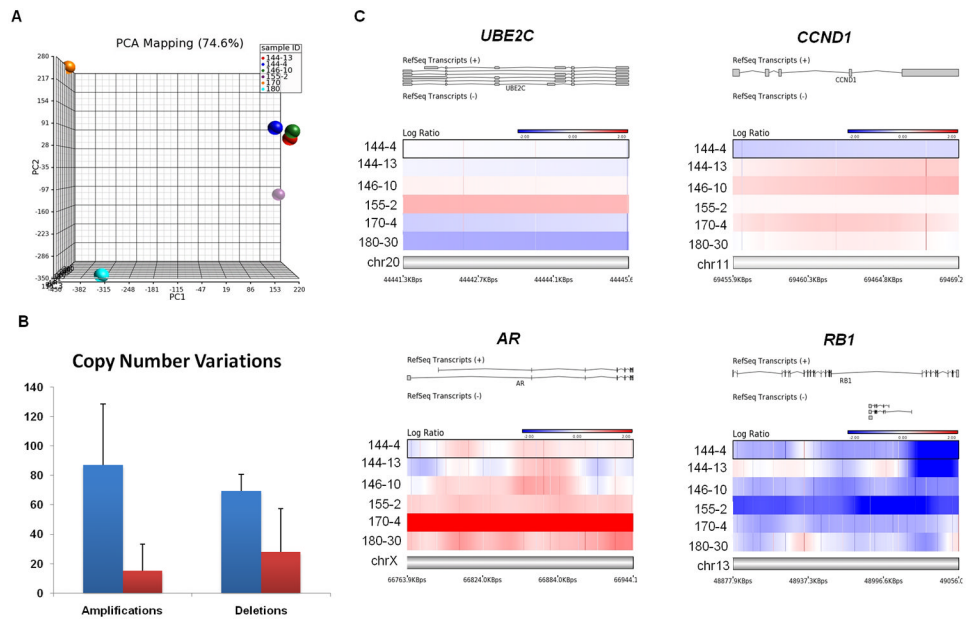
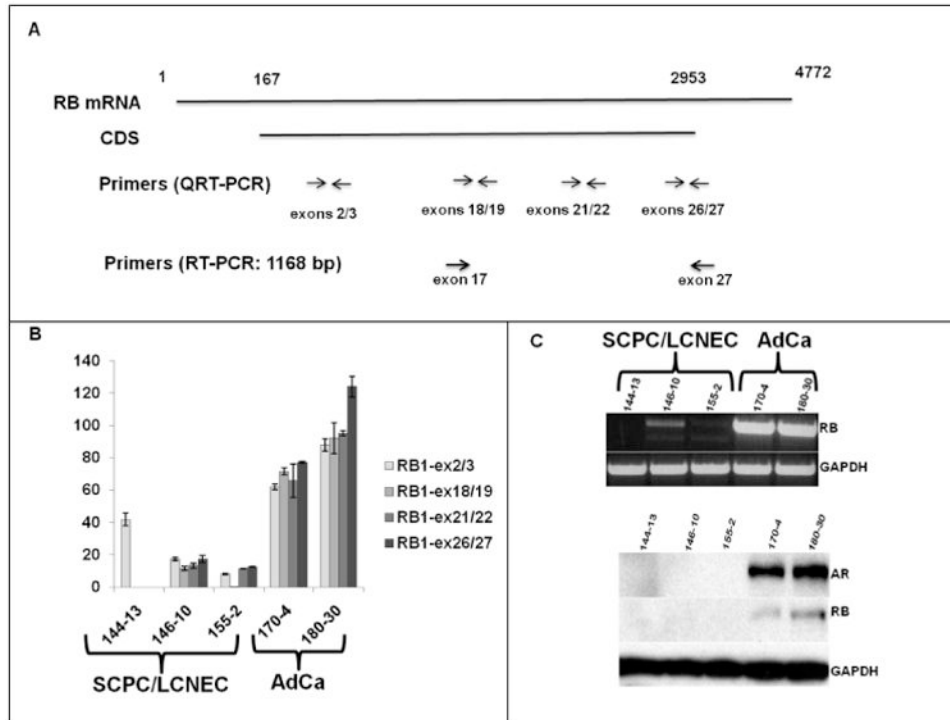


Figure 2. Results of aCGH analyses. A, Principal component analysis (PCA). B, Copy number variations (CNVs) in SCPC/LCNEC (blue) and adenocarcinoma (red) xenografts. C, aCGH of the *UBE2C*, *CCND1*, *AR*, and *RB1* genetic loci (SCPC/LCNEC xenografts are shown in blue and adenocarcinoma xenografts in red)

**Figure 3.**

A, Schematic representation of the RB mRNA and the primers used for the qRT-PCR and RT-PCR experiments. B, qRT-PCR results for *RB1* exons (ex) 2/3, 18/19, 21/22, and 26/27. C, Upper panel, RT-PCR results for exons 17 through 27; lower panel, Western blotting results for AR and RB expression, in which the bands correspond to the molecular weight of the full-length proteins (SCPC, small cell prostate carcinoma; LCNEC, large cell neuroendocrine carcinoma; AdCa, adenocarcinoma)

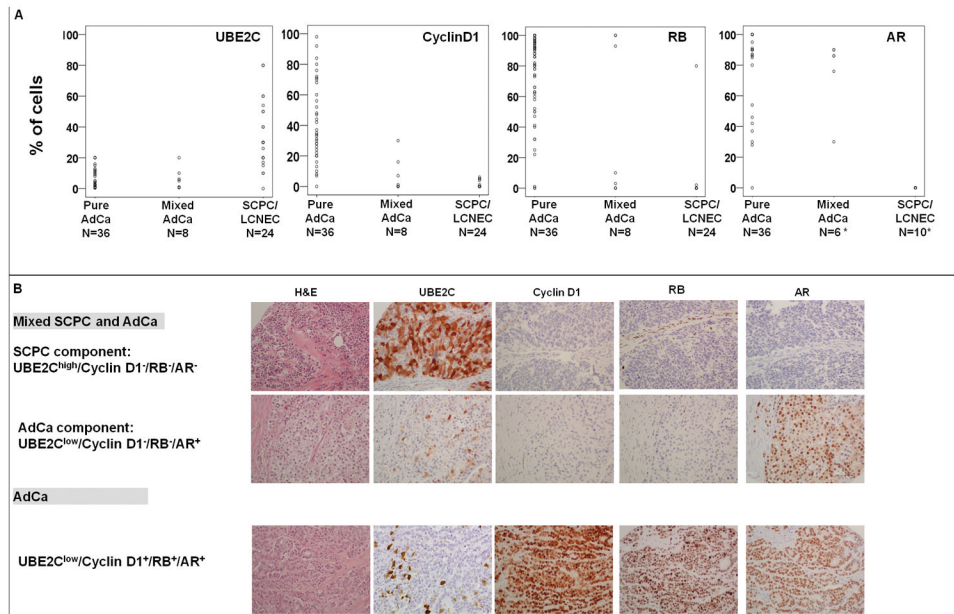


Figure 4. UBE2C, cyclin D1, RB, and AR protein expression in clinical CRPC tissues with SCPC/LCNEC prostate carcinoma or adenocarcinoma (AdCa) morphology. A, Scatter plots of UBE2C, cyclin D1, RB, and AR expression in the clinical CRPC samples. B, Representative images of hematoxylin and eosin (H&E)-stained and UBE2C-, cyclin D1-, Rb-, and AR-stained sections from a mixed SCPC-AdCa tumor and a pure AdCa tumor. (CRPC: castrate-resistant prostate carcinoma; SCPC, small cell prostate carcinoma; LCNEC, large cell neuroendocrine carcinoma; Original magnification, $\times 200$).

Table 1

The clinicopathologic features of the 8 xenograft donor patients.

Feature	MDA PCa Cell Line Designation ^d							
	79	117	130	144	146	155	170	180
At presentation								
Age (years)	61	60	65	68	74	72	63	72
PSA (ng/ml)	65	99	24	4.6	10.7	1.7	19.8	53
NBx morphology	AdCa	AdCa	AdCa	AdCa	AdCa	SCC	AdCa	AdCa
Gleason Score	4+4	5+5	4+4	4+3	3+4	-	9	9
Prior treatment								
Androgen ablation	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Radiation	Yes	No	Yes	Yes	Yes	No	No	No
Chemotherapy	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
At surgery								
Age (years)	67	64	76	72	78	72	64	74
Procedure	PE	CPX	CPX	PE	CPX	PE	CXPX	CXPX
Tissue morphology	AdCa	AdCa with NE diff	AdCa	Mixed AdCa SCPC and LCNEC	Mixed AdCa and SCPC	SCPC	AdCa	AdCa
GleasonScore	5+4	4+5	4+5	4+4	5+4	-	5+4	5+4
Pathologicstage	T4Nx	T4N1	T4N0	T4N1	T4N1	T4N0	T4N1	T4N1
Overall survival (years)	13.3	5.3	17.6	4.8	6.2	0.6	2.5	3.8
Site of tissue collection								
	RE	-1 ^b PR	PR	-4 PR	-10 BL	-2 BL	-1 PR	-11 BL
		-9 PR		-6 PR	-12 ^b BL	-9 BL	-4 PR	-14 BL
				-9 PR	-17 BL	-12 BL		-18 BL
				-11 BL	-20 BL	-16 PR		-21 BL
				-13 BL				-30 SV
				-17 BL				
				-20 RE				

MDA PCa Cell Line Designation ^a								
Feature	79	117	130	144	146	155	170	180
-23 RE								

^aNumbers indicated by “-” are cell sublines.

^bThese xenografts were unstable and were not developed any further.

Abbreviations: PSA, prostate-specific antigen; NBx, need lebiopsy; AdCa, adenocarcinoma; SCC, small-cell carcinoma; PE, pelvic exenteration; CPX, cystoprostatectomy; CXPX, cystoprostatectomy; NE diff, neuroendocrine differentiation; SCPC, small-cell prostate carcinoma; LCNEC, large-cell NE carcinoma; RE, rectal wall; PR, prostate; BL, bladder wall; SV, seminal vesicles.

Table 2
Histopathologic and immunohistochemical features of the xenografts and their respective donor tumors

Xenograft no.-subline no.	Diagnosis	AR ^a	PSA	Chromogranin A ^b	Synaptophysin ^b	UBE2C ^a	Cyclin D1 ^a	RB ^a	KI67 ^a
79									
<i>Donor Tumor</i>	<i>AdCa</i>	95	90	neg	neg	1	10	50	10
MDA PCa 79	AdCa	90	10 ^c	neg	neg	10	<1	100	15
117									
<i>Donor Tumor</i>	<i>AdCa with NE diff</i>	70	80	pos	pos	15	30	90	10
MDA PCa 117-9	AdCa with NE diff	95	10	pos	pos	20	50	100	10
130									
<i>Donor Tumor</i>	<i>AdCa</i>	95	80	neg	neg	20	70	100	10
MDA PCa 130	AdCa	80	40	neg	neg	30	70	95	10
144									
<i>Donor Tumor</i>	<i>AdCa</i>	90	40	neg	neg	5	20	5	30
	SCPC	<1	<1	pos	pos	60	0	0	95
	LCNEC	<1	5	pos	pos	40	0	N/A	95
MDA PCa 144-13	SCPC	0	0	pos	pos	60	0	0	85
MDA PCa 144-4	LCNEC	0	0	pos	pos	40	0	0	95
146									
<i>Donor Tumor</i>	<i>AdCa</i>	90	20	neg	neg	10	0	0	20
	SCPC	0	0	pos	pos	40	0	0	95
MDA PCa 146-10	SCPC	0	0	pos	pos	50	0	0	60
MDA PCa 146-17	SCPC	0	0	pos	pos	30	0	0	40
MDA PCa 146-20	SCPC	0	0	pos	pos	40	0	0	60
155									
<i>Donor Tumor</i>	<i>SCPC</i>	0	0	pos	pos	50	5	2	50

Xenograft no.-subline no.	Diagnosis	AR ^a	PSA	Chromogranin A ^b	Synaptophysin ^b	UBE2C ^a	Cyclin D1 ^a	RB ^a	KI67 ^a
MDA PCa 155-2	SCPC	0	0	pos	pos	50	0	0	30
MDA PCa 155-9	SCPC	0	0	pos	pos	60	0	0	40
MDA PCa 155-12	SCPC	0	0	pos	pos	40	0	0	40
MDA PCa 155-16	SCPC	0	0	pos	pos	70	0	0	50
170									
<i>Donor Tumor</i>	AdCa	95	20	neg	neg	10	70	100	25
MDA PCa 170-1	AdCa	100	60	neg	neg	25	80	100	30
MDA PCa 170-4	AdCa	95	15	neg	neg	15	75	100	55
180									
<i>Donor Tumor</i>	AdCa with NE diff	90	5	neg	pos	20	20	100	30
MDA PCa 180-11	AdCa with NE diff	95	0	neg	pos	25	30	100	70
MDA PCa 180-14	AdCa with NE diff	100	0	neg	pos	20	25	100	50
MDA PCa 180-18	AdCa with NE diff	100	5	neg	pos	15	50	100	60
MDA PCa 180-21	AdCa with NE diff	100	5	neg	pos	35	50	100	80
MDA PCa 180-30	AdCa with NE diff	95	5	neg	pos	20	50	100	50

^aPercentage of positive cells.

^bneg: 5% positive cells, pos:>5% positive cells

^cNote that some discrepancies between donor and xenograft immunohistochemical profiles exist, likely as a result of clonal selection in xenograft establishment. Nonetheless, all xenografts were shown to mirror the section of the donor tumor from which they were obtained.

Abbreviations: DT, donor tumor; AdCa, adenocarcinoma; NE, neuroendocrine; neg, negative; pos, positive; N/A, not available