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Cyclin D1 Loss Distinguishes Prostatic Small Cell Carcinoma from Most Prostatic Adenocarcinomas

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Abstract

Purpose—Small cell neuroendocrine differentiation in prostatic carcinoma is an increasingly common resistance mechanism to potent androgen deprivation therapy (ADT), but can be difficult to identify morphologically. We investigated whether cyclin D1 and p16 expression can inform on Rb functional status and distinguish small cell carcinoma from adenocarcinoma.

Experimental Design—We used gene expression data and immunohistochemistry to examine cyclin D1 and p16 levels in patient-derived xenografts (PDX), and prostatic small cell carcinoma and adenocarcinoma specimens.

Results—Using PDX, we show proof-of-concept that a high ratio of p16 to cyclin D1 gene expression reflects underlying Rb functional loss and distinguishes morphologically identified small cell carcinoma from prostatic adenocarcinoma in patient specimens (n=13 and 9, respectively). At the protein level cyclin D1, but not p16, was useful to distinguish small cell carcinoma from adenocarcinoma. Overall, 88% (36/41) of small cell carcinomas showed cyclin D1 loss by immunostaining compared to 2% (2/94) of Gleason score 7–10 primary adenocarcinomas at radical prostatectomy, 9% (4/44) of Gleason score 9–10 primary adenocarcinomas at needle biopsy, and 7% (8/115) of individual metastases from 39 patients at

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autopsy. Though rare adenocarcinomas showed cyclin D1 loss, many of these were associated with clinical features of small cell carcinoma, and in a cohort of men treated with adjuvant ADT who developed metastasis, lower cyclin D1 gene expression was associated with more rapid onset of metastasis and death.

Conclusions—Cyclin D1 loss identifies prostate tumors with small cell differentiation and may identify a small subset of adenocarcinomas with poor prognosis.

Keywords

Prostatic adenocarcinoma; small cell carcinoma; tumor suppressor; Cyclin D1; p16; RB1

Introduction

Small cell differentiation has become increasingly common in contemporary cohorts of metastatic castrate resistant prostate cancer (CRPC) (1–2). In its purest form, diagnosis of small cell carcinoma of the prostate has traditionally been a morphologic one (3). However there is increasing recognition that neuroendocrine differentiation in prostate cancer exists along a continuum, and hybrid or biphasic tumors with mixed features of small cell carcinoma and adenocarcinoma are commonly encountered (4). Further highlighting the potential difficulties in accurate pathologic diagnosis of prostatic small cell carcinoma, recent studies have identified a subset of patients with rapidly progressive prostate cancer and a small cell carcinoma-like clinical presentation, yet who lack classic morphologic features of small cell carcinoma in diagnostic biopsies (1, 4, 5). Despite these challenges in pathologic identification, it is clinically critical to distinguish small cell carcinoma from usual prostatic adenocarcinoma, both in the primary and metastatic settings. Small cell carcinomas are typically androgen receptor-negative or androgen receptor signaling-independent, thus platinum-based chemotherapeutic regimens are generally more effective for these patients than standard ADT (5, 6). Given these challenges in pathologic diagnosis, it would be useful to have sensitive and specific molecular markers of small cell neuroendocrine differentiation.

Loss of the retinoblastoma tumor suppressor gene (*RB1*) and p53 (*TP53*) are key molecular drivers of pulmonary small cell carcinoma (7, 8). Recently, our group showed that *RB1* loss occurs in the overwhelming majority of prostatic small cell carcinomas (9). *RB1* gene loss in small cell carcinoma can be detected via a simple immunohistochemical (IHC) assay for Rb protein and it is rarely seen in usual-type prostatic adenocarcinomas. However, Rb IHC may not detect cases where there is Rb functional loss, such as mutation, and in addition, this assay is not widely available in clinical pathology laboratories at this time. To address these issues, we sought to define a limited panel of widely available surrogate molecular markers that would reflect Rb functional status and distinguish small cell carcinoma from adenocarcinoma.

It has been known for two decades that cell lines and transgenic mice lacking functional Rb commonly show transcriptional feedback up-regulation of p16 and down-regulation of cyclin D1 (10–13). Similarly, small cell lung carcinomas and gastrointestinal neuroendocrine tumors show an inverse relationship between Rb/cyclin D1 expression and

p16 expression (14–16). In human papilloma virus (HPV) -related squamous cell carcinomas of the head and neck and cervix, Rb inactivation occurs via proteolytic degradation caused by the E7 viral antigen, resulting in p16-overexpression and loss of cyclin D1 expression which may be used as surrogate markers of HPV status in these sites (17, 18). A recent study of patient derived xenograft (PDX) models of prostatic small cell carcinoma demonstrated that cyclin D1 protein and mRNA is frequently lost in these tumors (19). Here, we investigated whether cyclin D1 loss and p16-overexpression reflect underlying Rb functional status in prostate tumors and can be exploited as molecular markers of small cell neuroendocrine differentiation in the prostate.

Materials and Methods

Patient sample selection

Tissue collection protocols were approved by the Johns Hopkins School of Medicine Institutional Review Board. A total of 48 morphologically identified small cell carcinoma cases were collected for IHC (n=41), gene expression profiling (n=13), or both. Four cases are described below with the metastatic samples. Forty-four cases were retrieved from the surgical pathology and consultation files of the Johns Hopkins Hospitals from 1994–2013, originating from 22 (50%) transurethral resections of the prostate, 21 (48%) bladder, prostate or rectal biopsies, and one (2%) radical prostatectomy. Twenty-five were previously described (9, 20). Prostatic origin of small cell carcinoma (vs secondary spread from a bladder or lung primary) was documented using the following criteria: a concurrent or prior history of prostatic acinar carcinoma, a positive PSA (prostate-specific antigen) immunostain and/or a documented negative cystoscopy. Although all 44 tissue samples were from prostate or contiguous sites representing local tumor (rather than distant metastatic) spread, due to the fact that the majority of cases were consultations, adequate clinical history was often not available to determine which small cell carcinomas arose in the context of prior hormonal therapy (t-NEPC) or which cases were previously treated with newer androgen deprivation therapies such as abiraterone and enzalutamide (9).

Two tissue microarrays (TMA) were manually constructed from 35 of the cases (2 additional cases were stained on standard slides, and 7 cases were used exclusively for gene expression analyses). A minimum of three 1.0 mm cores were punched from the small cell carcinoma component, the acinar carcinoma component (when present), and the paired benign prostatic tissue, with 3 to 18 cores from each patient represented on the array. 25% (11/44) of cases had a concurrent acinar carcinoma component present for evaluation.

In addition to the 11 concurrent acinar samples, 147 morphologically identified high grade adenocarcinoma cases unassociated with a small cell carcinoma component were assessed for either IHC (n=138) or gene expression (n=9). Ninety-four cases were radical prostatectomy specimens in previously described TMAs comprising several cohorts of high grade, high stage prostate tumors: 26% (24/94) were Gleason score 7, 14% (13/94) Gleason 8, 56% (53/94) Gleason 9, and 4% (4/94) Gleason 10 (21, 22). The remaining cases were biopsy specimens containing Gleason score 9–10 prostatic adenocarcinoma: 52 from prostate needle biopsy and one from bladder biopsy.

Finally, we also examined cyclin D1 and p16 expression in two series of metastatic CRPC sampled at rapid autopsy. The first series, from Johns Hopkins (23), included two to six metastases from 13 morphologically identified adenocarcinoma and 1 morphologically identified small cell carcinoma case, sampled in triplicate on a single TMA prepared for this study. The second series, from the University of Michigan (24), included one to five metastases from a total of 26 morphologically identified adenocarcinoma and 3 morphologically identified small cell carcinoma cases sampled in triplicate on two separate TMAs (25).

Patient derived xenografts

The LuCaP patient derived xenograft lines have been previously described and consist of prostate tumor tissues obtained from radical prostatectomies or rapid autopsies and serially passaged after grafting subcutaneously or in the subrenal capsule of 6–8 week old male SCID mice (26).

Gene expression profiling

Fresh frozen samples of 21 LuCaP PCa xenografts were processed to extract total RNA from cryostat sections and evaluated with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Gene expression profiling was performed according to the technical guidelines provided by the Agilent Whole Genome Expression Microarray system. Briefly, each xenograft RNA sample was linearly amplified for one round and labeled with Cy5, and cohybridized with a common reference RNA sample that was similarly amplified but labeled with Cy3. Agilent Feature Extraction software was used to grid and extract data. Raw microarray data was preprocessed through normexp background correction, Loess normalization, and Aquantile normalization using the Bioconductor package limma. To arrive at expression levels by gene, the normalized data was averaged over identical probes and then over commonly annotated probes, using gene annotations provided by Agilent from GEO database GPL6480. Processed Cy5/Cy3 ratios constituted the gene expression measures compared across samples.

Formalin fixed paraffin embedded (FFPE) samples of 13 small cell carcinomas (including 6 that were used in TMA described above and 7 not included in any other studies) and 9 Gleason 9–10 adenocarcinomas on needle biopsy were used for gene expression profiling. Tumor tissue was identified by accompanying H&E stained slide, and two 1.5 mm punches of the tissue block were obtained. RNA was extracted and hybridized to Human Exon 1.0 ST GeneChips (Affymetrix, Santa Clara, CA) as described previously (27), and microarray quality control was assessed using the Affymetrix Power Tools package. Probe set summarization and normalization were then performed by the SCAN algorithm (28), which normalizes arrays individually, based on ENTREZG custom CDF from the BrainArray project, followed by batch correction using the ComBat package to account for different processing times of the samples.

Immunohistochemistry

Immunostaining for cyclin D1 and p16 was performed on the Ventana Benchmark XT (cyclin D1) or Benchmark Ultra (p16) automated staining system (Ventana Medical

Systems, Tucson, AZ). A rabbit monoclonal antibody was used to detect cyclin D1 (pre-dilute, clone SP4-R, Ventana Medical Systems) and a mouse monoclonal antibody was used to detect p16 (pre-dilute, clone E6H4, CINtec®, Ventana Medical Systems). Following deparaffinization, antigen unmasking was performed by using either CC1 or CC1 Ultra (Ventana Medical Systems). Secondary detection was performed using the iView DAB Detection system for both antibodies. Rb immunostaining was performed manually as previously described using a mouse monoclonal anti-human Rb antibody (1: 100 dilution; Cell Signaling, Danvers, MA) (9).

Nuclear cyclin D1 and Rb immunostains were dichotomously scored by two urologic pathologists (CLM and TLL). A case was considered to have diffusely lost cyclin D1 or Rb protein if the sampled tumor showed loss (0+ staining) in >95% of tumor nuclei in one or more sampled tumor spots on the TMA. Cytoplasmic staining was not scored, but was generally concordant with nuclear staining. Positive nuclear staining in surrounding endothelial cells provided an internal control in most cases and a case was excluded if it lacked this endothelial staining. Needle biopsies (44 of high grade adenocarcinoma and 2 of small cell carcinoma) were scored as having cyclin D1 and/or Rb loss if a discrete focus of contiguous tumor showed loss of nuclear staining in >95% of tumor nuclei (Figure 3B), even if the remaining tumor showed retention of staining.

Nuclear p16 immunostaining was scored using a semi-quantitative scoring system, ranging from 0+ to 3+. 0+ scoring was recorded if the tumor was entirely negative for nuclear p16. 1+ scoring was recorded if 1–10% of cells showed strong nuclear and/or nuclear/cytoplasmic p16. 2+ was recorded if 11–70% of cells showed strong nuclear and/or nuclear/cytoplasmic p16. Finally, 3+ scoring was recorded if >70% of cells showed strong nuclear and/or nuclear/cytoplasmic p16. 3+ scoring was analogous to the current recommendations for what is considered positive p16 staining in lower anogenital tract lesions (29).

Gene expression analysis of high risk prostate adenocarcinoma at radical prostatectomy

Affymetrix Human Exon 1.0 ST gene expression data of 235 high risk radical prostatectomy samples (GSE62116) from the Mayo clinic was analyzed as previously described (30), with gene expression levels calculated by Affymetrix core-level summarization. Among the 235 patients, 33% were treated with adjuvant hormonal therapy, and of these, 45% subsequently developed clinical metastasis and 25% died of prostate cancer. Given a gene-expression-based quantity, Kaplan Meier survival curves were generated within various sub-cohorts for the top (high expression), middle (intermediate expression), and bottom (low expression) tertiles.

Results

Ratio of p16 (*CDKN2A*) to cyclin D1 (*CCND1*) gene expression level correlates with Rb status and distinguishes small cell carcinomas from high grade adenocarcinomas

To test whether mRNA expression ratio *CDKN2A/CCND1* is correlated with Rb functional status, we examined gene expression data from LuCaP patient derived xenografts (PDX) with known *RBI* genetic status (31). Data was available from 17 adenocarcinoma and 4

neuroendocrine xenografts, derived from 14 and 3 individual patients respectively. Neuroendocrine xenografts were previously classified as such on the basis of negative immunohistochemical staining for AR and PSA and positive immunohistochemical staining for synaptophysin and/or chromogranin (32). At least one neuroendocrine tumor (line 49) was also described as showing classic small cell carcinoma morphology (33). Consistent with our previous findings (9), all 4 (100%) neuroendocrine xenografts had homozygous loss of *RBI* by CGH analysis, compared to only 1 of 17 (6%) of the adenocarcinoma xenografts (line 86.2) (31). Using a previously described gene expression signature containing 159 genes with up-regulated expression following Rb functional loss (34), we calculated an Rb functional loss score for each xenograft as the average expression level of the signature genes, as described previously (31). Validating this Rb loss score, the xenografts with homozygous loss of *RBI* showed higher Rb loss scores compared to the xenografts with intact/hemizygous *RBI* (mean of 0.54 vs -0.16; $p=1.4e-5$; Figure 1A).

The previously described Rb loss gene expression signature did not include either p16 (*CDKN2A*) or cyclin D1 (*CCND1*). Since the mRNA expression ratio *CDKN2A/CCND1* has been shown to correlate with Rb functional status in cell lines and other tumor types (10–13, 35), we tested whether the ratio was correlated with *RBI* genetic status, the Rb functional loss score, and neuroendocrine differentiation in the LuCaP xenograft series (Figure 1A and 1B). There was a strong correlation between Rb loss score and *CDKN2A/CCND1* ratio ($r=0.75$), thus the *CDKN2A/CCND1* ratio also fairly accurately distinguished neuroendocrine carcinomas from adenocarcinomas (mean of 7.21 vs 2.31; $p=9.6e-8$). Largely similar results were obtained from examination of publicly available data from two other PDX datasets containing a mixture of prostatic small cell carcinoma and adenocarcinoma samples (19, 36) (Supplementary Figure S1). Of interest, in these latter two PDX datasets, the one adenocarcinoma xenograft with relatively high *CDKN2A/CCND1* ratio (sample 331) is an adenocarcinoma that transdifferentiates to neuroendocrine carcinoma (sample 331R) following androgen deprivation (36).

To test whether the *CDKN2A/CCND1* ratio and Rb functional loss gene expression scores were useful to distinguish small cell carcinomas from high grade adenocarcinomas in patient specimens, we profiled 13 morphologically identified small cell carcinomas and 9 Gleason 9–10 adenocarcinomas sampled on FFPE needle biopsy (Figure 1C). As with the xenografts, there was a strong correlation between Rb loss score and *CDKN2A/CCND1* ratio ($r=0.72$). Overall, both the Rb loss score and *CDKN2A/CCND1* ratio of the small cell carcinomas were significantly higher than that of the adenocarcinomas (mean of 0.10 vs -0.07; $p=0.006$; and mean of -0.61 vs -1.12; $p=0.013$; respectively). Interestingly, several of the small cell carcinomas segregated with the adenocarcinomas for *CDKN2A/CCND1* ratio and Rb loss score. We cannot exclude the possibility that this might be due to relatively low tumor cellularity in some of these cases which showed scant tumor on needle biopsy specimens. Similarly, one adenocarcinoma segregated with the small cell carcinoma cases. This sample was from a recently described patient in an active surveillance protocol who had a late progression event and developed castrate resistant prostate cancer within one year of ADT (37), anecdotally supporting the aggressive nature of this tumor despite an absence of small cell carcinoma morphology.

To confirm these results, we performed a similar analysis in publicly available gene expression data from metastatic tumor specimens (24). Here, we studied the University of Michigan rapid autopsy cohort, containing 2 small cell carcinomas and 29 non-small cell metastatic CRPC samples (Supplementary Figure S2). While the correlations between *RB1* copy number and *CDKN2A/CCND1* ratio and Rb functional loss scores in this set ($r=0.62$) were not as strong as in the datasets described above, the two small cell carcinomas still scored among the highest on both measures.

Loss of cyclin D1 protein is common in small cell carcinoma but uncommon in high grade primary adenocarcinoma and non-small cell metastatic CRPC

We next tested whether differential protein expression of cyclin D1 (and p16; see next section), widely available assays in most anatomic pathology laboratories, could be useful to distinguish prostatic small cell carcinoma from adenocarcinoma in FFPE tissues, and also compared with Rb protein expression.

Among morphologically identified prostatic small cell carcinomas, 88% (36/41) showed complete loss of cyclin D1 protein by immunostaining, and 92% (36/39) showed Rb loss, similar to our previously published Rb data (which included 25/41 of these cases) (Figure 2, Table 1). For comparison, we examined various groups of high grade adenocarcinomas from different settings (Figure 3, Table 1). As detailed below, cyclin D1 protein loss occurred in these groups at low rates (2%–18%, overall 6%) that were also consistently less frequent than the Rb protein loss rates (10%–36%, overall 14%) established in previous studies. Thus cyclin D1 loss, compared to Rb loss, appears to be almost as sensitive and more specific as a potential marker of small cell carcinoma in high grade prostatic tumors.

Despite their infrequency in adenocarcinoma, cyclin D1 loss and Rb loss remained highly associated with each other in this group ($p=6.6e-10$, Table 2), and the joint loss phenotype behaved similarly to the cyclin D1 loss phenotype overall (Table 1). Notably, cyclin D1-negative tumors tended to have concurrent Rb loss independent of type: 80% (13/16) of cyclin D1-negative adenocarcinomas were also Rb-negative, which was not significantly different than the Rb loss frequency (94%, 32/34) in cyclin D1-negative small cell carcinomas ($p=0.31$ by Fisher's exact test). By contrast among Rb-negative tumors, the frequency of concurrent cyclin D1 loss was quite different between types: only 34% (13/38) of the Rb-negative primary or metastatic adenocarcinomas had concurrent cyclin D1 loss, compared to 89% (32/36) of the Rb-negative small cell carcinomas ($p=1.2e-6$ by Fisher's exact test). In other words, the greatest discordance between cyclin D1 loss and Rb loss occurred in adenocarcinomas, where cyclin D1-negative tumors were usually Rb-negative but not vice versa (Figure 3B).

There was slight variation and a few interesting cases among the various groups of adenocarcinomas. Of all groups, the rate of cyclin D1 loss was highest (18% or 2/11) in the adenocarcinomas containing small cell carcinoma within the same specimen, although only 11 such cases were available. In the radical prostatectomy group, consisting of 94 Gleason 7–10 primary adenocarcinomas on TMAs, only 2% (2/94) showed cyclin D1 loss. Both cases were Gleason 9 with cyclin D1 loss seen in all sampled tumor spots, and one case had concurrent Rb protein loss. Because radical prostatectomy cases are generally highly

selected for lower stage tumors potentially amenable to surgical cure, we also examined 44 Gleason 9–10 primary adenocarcinomas sampled on prostate biopsy. In this group, 9% (4/44) showed cyclin D1 loss, with 3 of 4 negative cases showing only focal areas of cyclin D1 loss, and Rb protein was concurrently lost in all 4 of these cases. Of interest, one of the 4 adenocarcinomas with focal Rb and cyclin D1 loss developed a synchronous small cell carcinoma metastasis in the liver, though we did not know of this at the time we selected the case for immunostaining (Figure 3C). Finally, we examined cyclin D1 loss in non-small cell (by morphology) metastatic CRPC sampled from two rapid autopsy series (Figure 3D). Examining all metastases together, 7% (8/115) of individual metastases from 39 patients showed cyclin D1 loss, and 6 of the 8 had concurrent Rb loss. Overall, 15% (6/39) of patients had at least one metastasis with cyclin D1 loss. As we reported previously for Rb protein loss, there was frequent inter-metastatic heterogeneity for cyclin D1 protein loss within a given patient: 66% (4/6) of patients with a metastasis showing cyclin D1 loss had cyclin D1 loss in only one of 3 to 5 sampled metastases (the other patients were cyclin D1-negative in 1/1 and 3/3 sampled metastases).

Effect of androgen deprivation on cyclin D1/p16/Rb expression

The adenocarcinomas described above were largely hormone-naïve at time of biopsy or radical prostatectomy. By contrast, the metastatic samples were post-hormone therapy, as were the primary tumor samples used to establish the CRPC xenografts in (19). Though clinical data is incomplete on the small cell carcinoma samples, at least a subset were also exposed to ADT prior to biopsy. Since cyclin D1 and Rb loss were much more common in the latter groups compared to the untreated primary samples, this raises the question of whether the higher rates of cyclin D1 loss in the metastatic and small cell samples could reflect prior exposure to ADT instead of, or in addition to, underlying Rb status. To assess whether there is a direct or short-term effect of ADT on expression of these markers, we examined published expression data from the androgen-dependent LNCaP cell line after 48 hours of simulated androgen withdrawal compared to steady state or compared to androgen deprivation followed by DHT stimulation (38). *In vitro*, gene expression of cyclin D1, p16 and Rb were relatively unchanged in response to androgen modulation, although Rb functional loss score decreased after ADT, likely reflective of response to treatment and captured within the cell proliferation component of the Rb loss gene signature (Supplementary Figure S3) (34). The positive correlation between *CDKN2A/CCND1* and Rb loss score also deteriorated. Next, we examined several datasets of *in vivo* primary adenocarcinomas treated with androgen deprivation. A recent study analyzed biopsies of 7 patients both before and 22 weeks after ADT initiation, and similar to the LNCaP data, *CCND1*, *CDKN2A*, and *RB1* were not differentially expressed while Rb functional loss score decreased for each patient and de-correlated from *CDKN2A/CCND1* (Supplementary Figure S3) (39). Past studies have also compared cohorts of patients receiving neoadjuvant ADT (3 months in 2 studies, 1–9 months in the other study) versus no ADT prior to radical prostatectomy, and *CCND1*, *CDKN2A*, and *RB1* were not reported in any of the lists of differentially expressed genes (40–42). Finally, we examined 29 adenocarcinomas treated with neo-adjuvant hormonal therapy prior to radical prostatectomy and found only one case (3%) with focal cyclin D1 loss, a rate of loss roughly equivalent to that seen in untreated adenocarcinomas (data not shown). Taken together, these data do not support a major direct

or indirect effect of ADT on expression of cyclin D1, Rb and/or p16 *in vitro* or *in vivo* for typical prostatic adenocarcinomas. However, we cannot rule out the possibility that ADT may contribute to selective growth of rare Rb deficient adenocarcinomas, since Rb-negative prostate cells are resistant to the cytostatic effects of ADT *in vitro* (43). Along these lines, the Vancouver adenocarcinoma PDX #331 which reliably transdifferentiates several months post-castration to a neuroendocrine phenotype #331R was shown to be hemizygous for *RBI* with a frame-preserving insertion mutation in the remaining allele, and its Rb functional loss score increased markedly upon transdifferentiation while appearing relatively unchanged in the immediate post-castration period (44). Both *CCND1* down-regulation and *CDKN2A* up-regulation were also included among a list of gene expression trends potentially involved in transdifferentiation, whereas no such trend was observed for the other castrate resistant model pair, #331B and #331BR, representative of more typical CRPC (36) (Supplementary Figure S3). However in follow-up analysis, *CCND1* and *CDKN2A* did not appear to exhibit significant expression trends across the post-castration time series (44). Additional larger studies would be desirable to better characterize the temporal behavior of these markers *in vivo* under ADT.

p16 protein expression is not useful for distinguishing prostatic small cell carcinoma from adenocarcinoma

Because the gene expression data suggested that p16 over-expression might be a useful marker of Rb genetic and functional loss, we also examined p16 expression in the small cell carcinomas and primary and metastatic adenocarcinomas described above (Table 1, Figures 2 and 3). Though expression was generally higher in small cell carcinoma than in adenocarcinoma, p16 protein showed a wide range of expression among all specimens. Overall, only 51% (20/39) of small cell carcinomas showed strong nuclear or nuclear and cytoplasmic over-expression of p16 protein in more than 70% of cells (3+ staining). Of note, 100% (20/20) of these cases showed underlying Rb protein loss and 85% (17/20) showed underlying cyclin D1 loss. Interestingly, we identified one small cell carcinoma case where p16 expression was entirely lost, suggesting the possibility of underlying genetic deletion of the *CDKN2A* locus, an event which has been reported rarely in prostatic small cell carcinomas (45) and also occurs in a small percentage of adenocarcinomas (Figure 2C). This unusual case showed high expression of cyclin D1 as well as very high expression of Rb, suggesting an intact *RBI* locus and underlying Rb inactivation due to p16 loss via hyperphosphorylation (46).

Among primary adenocarcinomas sampled on radical prostatectomy and needle biopsy as described above, 19% (25/133) showed p16 over-expression, of which 80% (20/25) were from the cohort of 44 Gleason 9–10 adenocarcinomas sampled on needle biopsy. Similarly, 27% (3/11) of adenocarcinomas occurring with concurrent small cell carcinoma showed p16 over-expression. In contrast, only 10% (12/115) of non-small cell CRPC cases showed p16 over-expression at the protein level. Among primary adenocarcinomas, p16 over-expression showed poor correlation with underlying Rb and cyclin D1 protein status, with only 20% (5/25) of the p16-over-expressing tumors showing underlying Rb protein loss and 16% (4/25) showing underlying cyclin D1 loss.

High risk primary adenocarcinomas with *CCND1* loss have rapid development of metastasis and death in the context of adjuvant ADT

Overall, we found that a small minority of primary and metastatic adenocarcinomas lost cyclin D1 protein or showed a markedly increased *CDKN2A/CCND1* ratio, similar to small cell carcinoma. Anecdotal evidence described above suggested that these tumors may be clinically distinct from ordinary adenocarcinomas, with a more rapid progression to CRPC and death, such as has been described for a rare group of “anaplastic” adenocarcinomas with small cell-like clinical presentation (1, 4, 5). Thus, we hypothesized that elevated *CDKN2A* and/or low *CCND1* expression in morphologically identified adenocarcinomas might identify a rare group of aggressive, “small cell-like” carcinomas that would be relatively unresponsive to ADT. We used gene expression data from a previously described cohort of patients with high risk primary adenocarcinomas treated with radical prostatectomy with or without adjuvant ADT (30). Focusing on the subset of patients who developed metastasis, patients in the bottom tertile of *CCND1* expression experienced more rapid metastasis ($p=0.004$) and prostate cancer specific death ($p=0.03$) compared to intermediate or high *CCND1* expression, and this association was seen only in the ADT-treated group (Figure 4). In contrast, *CDKN2A* levels were less informative, though high *CDKN2A/CCND1* ratio was associated with decreased time to metastasis in the ADT group, largely driven by low *CCND1* (Supplementary Figure S4). Similarly, Rb functional loss score also was not associated with prognosis (data not shown).

Discussion

There is an emerging appreciation that prostatic small cell carcinoma exists on a morphologic spectrum, spanning cases with classic oat cell morphology (which may be easily diagnosed on H&E staining) to those with hybrid or mixed features with adenocarcinoma (4). Moreover, in the clinic, oncologists may encounter cases lacking the classic morphologic features of small cell carcinoma, yet behaving clinically as such, with a preponderance of visceral rather than bony metastases and a relatively low PSA for the extent of the metastatic burden (1, 4, 5). Because these cases may be insensitive to ADT from the outset and respond in the short term to platinum-based chemotherapeutic regimens, accurate pathologic diagnosis is critical. Diffuse positivity for neuroendocrine markers (such as synaptophysin, chromogranin and CD56) and negativity for androgen signaling axis markers (such as PSA and AR) have traditionally been the mainstay of immunophenotypic diagnosis of small cell carcinoma (3, 47). However, neuroendocrine markers can be negative in 20% of small cell carcinomas and focally positive in up to 100% of adenocarcinomas in some series (48). Similarly, up to 20% of small cell carcinomas can express androgen receptor and/or downstream androgen signaling targets (20, 47). Thus, additional molecular and immunohistochemical markers would be extremely helpful to support morphologic diagnosis of prostatic small cell carcinoma in routine clinical practice.

We recently demonstrated that Rb loss is nearly universal in small cell prostate carcinoma and is an excellent marker to distinguish small cell carcinoma from prostatic adenocarcinoma (9). Here, we have extended this work to show that expression of cyclin D1, an immunostain widely available in clinical pathology laboratories, is highly correlated

with underlying Rb genetic and functional status in prostate cancer, and thus helps to distinguish prostatic small cell carcinoma from adenocarcinoma. Building on prior studies in cancer cell lines (13), HPV-related carcinomas (17, 18) and prostatic patient derived xenografts (PDX) (19), we first demonstrated that a high ratio of *CDKN2A* (p16) to *CCND1* (cyclin D1) mRNA reflects *RB1* gene loss and is tightly correlated in prostate cancer with high scores on a previously published 159 gene signature of Rb functional loss (34). Consistent with this RNA expression data, at the protein level, cyclin D1 loss was specific for underlying loss of Rb immunostaining across all prostate tumor types studied. Rb loss occurred in 94% (32/34) of small cell carcinomas with cyclin D1 loss, 83% (5/6) of high grade adenocarcinomas with cyclin D1 loss, and 75% (6/8) of metastatic CRPC tumors with cyclin D1 loss.

Because Rb loss is nearly universal in small cell carcinoma and relatively rare in adenocarcinoma, a high *CDKN2A/CCND1* ratio was sufficient to distinguish prostatic small cell carcinomas from adenocarcinomas with few exceptions. Consistent with this, *CDKN2A* and *CCND1* both appear in recently published gene expression signatures for neuroendocrine prostate cancer (19, 36). Taken together, these gene expression data strongly predicted that loss of cyclin D1 protein and over-expression of p16 protein might be useful for diagnosis of small cell carcinoma and should be highly correlated with underlying Rb protein loss. Indeed, 88% (36/41) of morphologically diagnosed prostatic small cell carcinomas showed complete cyclin D1 loss compared to 4% (6/138) of high grade primary adenocarcinomas and 7% (8/115) of non-small cell castrate resistant metastases (CRPC). One important limitation of our dataset is that the small cell carcinomas studied were poorly clinically annotated, and many were relatively old. Thus, our cohort of small cell carcinoma may not be entirely representative of more contemporary small cell tumors developing in the context of newer androgen deprivation therapies such as enzalutamide and abiraterone. However, if validated in additional larger studies in contemporary cohorts, these results suggest that cyclin D1 may be a useful adjunct marker to support a diagnosis of prostatic small cell carcinoma.

Though cyclin D1 loss occurred in the context of concurrent Rb protein loss as expected in the majority of both small cell carcinomas and adenocarcinomas, the fraction of cases with Rb loss that had concurrent cyclin D1 loss was quite different in the two groups of tumors. Proportionally, there were significantly more adenocarcinomas showing Rb loss but not cyclin D1 loss (Figure 3B). Given that Rb loss is well known to result in feedback down-regulation of cyclin D1 at the transcriptional level (11, 35), the underlying molecular mechanism for this apparent lack of feedback in some adenocarcinomas remains unclear. Of interest, similar results can be seen at the transcriptional level in the non-small cell CRPC metastases from the University of Michigan (Supplementary Figure S2), where homozygous *RB1* gene loss occurs in 5 non-small cell metastases (5/28 or 18%), of which 60% (3/5; #3, #10 and #25) lack substantially elevated *CDKN2A/CCND1* or functional Rb loss expression scores. Though it is possible that this discrepancy could reflect the effects of chemotherapeutic treatment on cell cycle parameters in the CRPC setting, the fact that we see a similar phenomenon at the protein level in untreated adenocarcinomas (where 10/15 or 67% have Rb protein loss in the absence of cyclin D1 loss) suggests that this may not be the

full explanation. Interestingly, in transgenic mice with retinal *RB1* loss, the feedback down-regulation of cyclin D1 expression is more potent when *RB1* is lost concurrently with loss of another *RB1* family member, such as p105 or p130 (*RBL1* and *RBL2*, respectively) (12). Thus, it is possible that down-regulated expression of other Rb family or other cell cycle genes in small cell carcinomas may differentially amplify the effects of Rb loss on cyclin D1 expression.

Though p16 protein levels were frequently higher in small cell carcinomas than in adenocarcinomas as predicted from the gene expression data, the variability in staining for this marker across diagnostic categories limited its clinical utility at the protein level. The underlying cause for this discrepancy is unclear, but it may in part be due to pre-analytic variables. Tissue fixation conditions, processing protocols and/or block age can all affect immunostaining results in an unpredictable and antigen-specific fashion. In addition, there may be rare cases of small cell carcinoma in which functional inactivation of Rb takes place via loss of p16 expression (45), further undermining the utility of p16 over-expression for identifying small cell carcinomas. Though we did not observe biallelic loss of *CDKN2A* in our previously published screen of 12 prostatic small cell carcinomas, this is a common cause of Rb inactivation in other tumor types and genetic loss of *CDKN2A* is known to occur in a small subset of prostatic adenocarcinomas. In the current study, we did identify one small cell carcinoma case with likely *CDKN2A* loss occurring in the context of intact Rb protein expression, suggesting that it may occur rarely in this context.

One theme emerging from these studies is that there clearly exists a rare group of high grade morphologic adenocarcinomas that molecularly resemble small cell carcinomas with respect to Rb functional status and cyclin D1 expression. While these tumors generally represent fewer than 10% of all primary and metastatic adenocarcinomas analyzed in both our gene expression and immunohistochemical studies, a key question remaining is whether these may behave clinically like small cell carcinoma (5) or whether they may be more likely to transdifferentiate into full-blown small cell carcinoma in the setting of ADT (36). In support of this possibility, the small sample of adenocarcinomas occurring with concurrent small cell carcinoma in this study did show somewhat higher Rb and cyclin D1 loss, suggesting a clonal relationship with the concurrent small cell carcinoma component and a shared profile of molecular aberrations in some cases (49). Additionally, anecdotal evidence from the current study and others suggests that morphologically unremarkable adenocarcinomas with Rb and/or cyclin D1 loss do have an aggressive clinical course, with concurrent development of small cell carcinoma metastases and/or rapid development of CRPC. In analysis of the Vancouver PDX series, one adenocarcinoma with a higher ratio of *CDKN2A/CCND1* has been described to transdifferentiate to neuroendocrine carcinoma upon androgen deprivation (36) (sample 331, Supplementary Figure S1). Among the Gleason 9–10 primary adenocarcinomas that we profiled for *CDKN2A/CCND1* expression ratio, the only one that clustered with the small cell carcinoma samples was previously reported to have developed into CRPC within 1 year of diagnosis (37). Finally, one of the four Gleason 9–10 primary adenocarcinomas with cyclin D1 protein loss was associated with a synchronous small cell carcinoma metastasis.

Consistent with these anecdotal data, a preliminary analysis of a high risk surgical cohort of adenocarcinomas showed that among patients who received adjuvant ADT and still developed metastasis, lower expression of *CCND1* was associated with a more rapid onset of metastasis and death, with a median interval of only 2 years between radical prostatectomy and metastasis. Interestingly, a similar association was not seen for patients who did not receive ADT, suggesting ADT may not be an optimal treatment option for tumors with cyclin D1 loss. Because adjuvant ADT was not randomly assigned in this cohort, we cannot exclude the possibility that the ADT-treated cohort represents a clinically higher risk group which may simply contain more “small cell-like” adenocarcinomas. However, this finding also raises the provocative question of whether adjuvant ADT may have the detrimental effect of creating selection pressure for the transdifferentiation of neuroendocrine-like carcinomas, particularly among tumors with the right molecular profile, such as Rb and cyclin D1 loss, resulting in rapidly poor outcomes. These findings raise the possibility that Rb and/or cyclin D loss may be useful to help select patients who might most benefit from early chemotherapy.

Ultimately, our data suggest that molecular markers such Rb loss and cyclin D1 may have a wider utility as predictive biomarkers, guiding treatment decisions for adenocarcinomas in addition to identifying neuroendocrine tumors. Despite evidence presented above that adenocarcinomas with cyclin D1 loss have an aggressive clinical course similar to small cell carcinomas, a major limitation is that these patients did not received a standardized treatment regimen within the context of a clinical trial and this precludes a more formal analysis of whether cyclin D1 might predict for response to specific therapies. Given the relatively low rate of cyclin D1/Rb loss among even the very high grade adenocarcinomas studied herein, much larger studies will be required to address this issue within the context of ongoing clinical trials. Along these lines, a recent report showed that the loss of Rb was a biomarker of enhanced response to cabazitaxel (50).

In conclusion, we have shown that a high ratio of *CDKN2A* to *CCND1* expression and loss of cyclin D1 protein expression are highly correlated with underlying Rb functional loss and distinguish morphologically identified small cell carcinoma of the prostate from adenocarcinoma in most cases, in both the primary and metastatic settings. These molecular markers may be useful to identify prostate tumors with small cell neuroendocrine differentiation in pathologic practice, and, if validated in future studies, may serve as prognostic and/or predictive biomarkers for response to ADT and/or chemotherapy in prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

Small cell neuroendocrine carcinoma of the prostate is increasingly common in the context of potent androgen deprivation therapies (ADT). Pathologic identification of small cell carcinoma can be challenging and is important for appropriate clinical management of these patients. Thus identifying ancillary molecular markers of small cell differentiation to aid in pathologic diagnosis is critical. Loss of the *RB1* tumor suppressor gene is a key driver event in prostatic small cell carcinoma, and is rare in prostatic adenocarcinoma. Here, we demonstrate that at the gene expression level, loss of cyclin D1 and over-expression of p16 provide surrogate markers of Rb functional loss and distinguish adenocarcinoma from small cell carcinoma of the prostate. Further, at the protein level, an immunohistochemical assay for cyclin D1 (widely available in most diagnostic pathology labs) identifies prostate tumors with small cell differentiation and may identify a small subset of adenocarcinomas with poor prognosis.

Figure 1. The ratio of *CDKN2A/CCND1* reflects Rb functional and genetic status and distinguishes small cell carcinoma from prostatic adenocarcinoma

(A) Gene expression data from LuCaP patient derived xenografts (PDX) demonstrates that high *CDKN2A/CCND1* correlates with a previously described functional Rb loss gene expression score ($r=0.75$). The highest scoring tumors on both scores are predominantly neuroendocrine carcinomas (NE) with *RB1* homozygous loss, while the adenocarcinomas (AdCa) show lower *CDKN2A/CCND1* scores. The mean *CDKN2A/CCND1* ratio of neuroendocrine xenografts was 7.21, substantially higher than that of the adenocarcinoma xenografts 2.31 ($p=9.6e-8$). One adenocarcinoma xenograft, #86, shows homozygous *RB1* loss and clusters with the neuroendocrine carcinomas. (B) *CCND1* and *CDKN2A* expression levels demonstrate high *CDKN2A* and low *CCND1* expression among samples with homozygous *RB1* loss. (C) Gene expression data from formalin fixed paraffin embedded patient samples demonstrates a similar pattern of higher *CDKN2A/CCND1* ratios and Rb functional loss scores in the small cell carcinomas compared to the adenocarcinoma samples. The mean *CDKN2A/CCND1* ratio for small cell carcinoma was -0.61 vs -1.12 for adenocarcinoma ($p=0.013$). One of the outlier adenocarcinoma samples (arrow) was

previously described (38) and developed castrate resistant prostate cancer (CRPC) within one year of ADT therapy. **(D)** *CCND1* and *CDKN2A* expression levels demonstrate higher *CDKN2A* and lower *CCND1* expression among small cell carcinoma cases in general compared to adenocarcinoma cases. The outlier adenocarcinoma described in (C) is identified with an arrow.

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Figure 2. Morphologically identified small cell carcinoma shows cyclin D1 protein loss and generally high expression of p16 protein

(A) Immunostaining of a morphologically identified small cell carcinoma on TMA demonstrates total cyclin D1 and Rb protein loss, with high (3+) p16 expression. Note the presence of cyclin D1- and Rb-positive endothelial cells as internal positive controls (inset, arrows). (B) Mixed small cell carcinoma and concurrent adenocarcinoma sample shows loss of cyclin D1 and Rb in small cell component with retention in adenocarcinoma component. p16 is highly expressed in small cell carcinoma (3+ staining) while largely negative in adenocarcinoma component. NKX3.1 immunostaining highlights adenocarcinoma component, while this androgen-regulated prostate-specific marker is lost in the small cell component as previously reported (7). (C) Rare morphologically identified small cell carcinoma with strong cyclin D1 and Rb immunostaining and complete loss of p16 protein expression (note positive staining in fibromuscular stromal cells as internal positive control).

The high level of Rb protein expression (compare to Rb expression in adenocarcinoma in (B) likely indicates highly phosphorylated Rb.

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Figure 3. Morphologically identified primary and metastatic adenocarcinoma shows intact cyclin D1 and variable expression of p16 protein

(A) Immunostaining of a high grade primary adenocarcinoma on TMA demonstrates high cyclin D1 expression, and low level (1+) p16 expression and intact Rb staining. (B) Immunostaining of a rare high grade primary adenocarcinoma on needle biopsy demonstrates loss of Rb immunostaining with intact cyclin D1 staining, though focally downregulated, and moderate p16 (2+) immunostaining. (C) Immunostaining of a rare high grade primary adenocarcinoma on needle biopsy demonstrates two adjacent and morphologically distinct tumor components (inset), with loss of cyclin D1 immunostaining in one component (bottom panels) with high p16 expression (1+–2+) compared to adjacent

component with intact cyclin D1 immunostaining and absence of p16 immunostaining (0–1+). This patient had a synchronous small cell carcinoma liver metastasis, though neither component of the primary tumor was morphologically consistent with small cell carcinoma, showing abundant cytoplasm and some nucleoli (insets). **(D)** Metastatic non-small cell castrate resistant prostate cancer (CRPC) with intact cyclin D1 expression, low p16 expression (1+) and intact Rb protein expression.

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Figure 4. High risk adenocarcinomas with low cyclin D1 levels are associated with more rapid metastasis in the setting of androgen deprivation therapy (ADT)

Kaplan Meier curves of metastasis-free and prostate cancer specific mortality (PCSM)-free survival demonstrates decreased time to metastasis and death among patients in bottom tertile of *CCND1* expression compared to intermediate or high *CCND1* expression in group treated with ADT. This association is not present in the cohort that did not receive ADT.

Frequency of cyclin D1 and Rb protein over-expression in prostate tumors

Table 1

	n	cyclin D1-negative	Rb-negative	p16-positive	Rb-negative and cyclin D1-negative
Small cell carcinoma	41	88%	92%	51%	82%
Adenocarcinoma occurring with small cell carcinoma	11	18%	36%	27%	18%
Primary adenocarcinoma	138	4%	11%	19%	4%
CRPC metastases	115	7%	17%	10%	5%

Table 2

Cyclin D1 and Rb protein loss status from different sample groups

	Rb-negative	Rb-positive	p
Small cell carcinoma			
cyclin D1-negative	32	2	0.34
cyclin D1-positive	4	1	
AdCa occurring with SC			
cyclin D1-negative	2	0	0.11
cyclin D1-positive	2	7	
Prim AdCa from RP (GS 7–10)			
cyclin D1-negative	1	1	0.18
cyclin D1-positive	8	84	
Prim AdCa from Bx (GS 9–10)			
cyclin D1-negative	4	0	0.0001
cyclin D1-positive	2	37	
Met AdCa from autopsy (CRPC)			
cyclin D1-negative	6	2	0.0002
cyclin D1-positive	13	94	
AdCa (all together)			
cyclin D1-negative	13	3	6.6e-10
cyclin D1-positive	25	222	

AdCa: adenocarcinoma, SC: small cell carcinoma, Prim: primary, Met: metastatic GS: Gleason score, CRPC: castrate resistant prostate cancer, p-val by Fisher exact test