THEMATIC REVIEW

Therapy considerations in neuroendocrine prostate cancer: what next?

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Abstract

Lineage plasticity and histologic transformation to small cell neuroendocrine prostate cancer (NEPC) is an increasingly recognized mechanism of treatment resistance in advanced prostate cancer. This is associated with aggressive clinical features and poor prognosis. Recent work has identified genomic, epigenomic, and transcriptome changes that distinguish NEPC from prostate adenocarcinoma, pointing to new mechanisms and therapeutic targets. Treatment-related NEPC arises clonally from prostate adenocarcinoma during the course of disease progression, retaining early genomic events and acquiring new molecular features that lead to tumor proliferation independent of androgen receptor activity, and ultimately demonstrating a lineage switch from a luminal prostate cancer phenotype to a small cell neuroendocrine carcinoma. Identifying the subset of prostate tumors most vulnerable to lineage plasticity and developing strategies for earlier detection and intervention for patients with NEPC may ultimately improve prognosis. Clinical trials focused on drug targeting of the lineage plasticity process and/or NEPC will require careful patient selection. Here, we review emerging targets and discuss biomarker considerations that may be informative for the design of future clinical studies.

Key Words

- prostate cancer
- lineage plasticity
- neuroendocrine prostate cancer
- epigenetics
- biomarkers

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Introduction

The treatment landscape of advanced prostate cancer continues to evolve. Drugs with varied mechanisms of action now being used in earlier disease settings, and patients are living longer with longer exposure to systemic therapies. However, systemic therapies are not curative, and the treatment-resistant state remains a major medical problem. Resistance mechanisms in prostate cancer are diverse, dependent on the biologic pathways targeted and typically occur in the context of activated androgen receptor (AR)-signaling. While the AR is the main key driver of prostate cancer initiation and progression, a subset of prostate cancers either start relatively AR-independent or, more commonly, they acquire AR-independence during the course of their disease. The frequency of AR-independence has been reported in up to 15–20% of CRPC tumors after treatment with potent AR-targeted drugs (e.g. abiraterone, enzalutamide) (Bluemn *et al.* 2017, Aggarwal *et al.* 2018, Abida *et al.* 2019). Developing effective therapies for AR-independent prostate cancer is particularly challenging, as there is heterogeneity even within this subset and continued clonal and sub-clonal evolution of castration-resistant tumors as they progress. One increasingly recognized mechanism of AR independence is lineage plasticity, a process that involves the acquisition

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of alternative lineage programs to drive cancer growth and progression (Beltran *et al.* 2019) (Fig. 1). These alternative programs, commonly of neuronal or neuroendocrine lineage, are pointing to acquired pathways and targets that may be exploited therapeutically. Clinically, lineage plasticity may manifest as a histologic transformation from a prostate adenocarcinoma to a small cell neuroendocrine carcinoma (Beltran *et al.* 2011, Bluemn *et al.* 2017, Aggarwal *et al.* 2018). As new drugs enter the clinic for neuroendocrine prostate cancer, patient selection will be critical.

Missing link: why we need biomarkers

With increased recognition of diverse castration-resistant prostate cancer (CRPC) subtypes, metastatic biopsies are increasingly being performed. Genomic sequencing to look for actionable targets (e.g. DNA repair alterations) is now considered a standard of care. Metastatic biopsies have also identified morphologic heterogeneity within and across patients with CRPC. While most CRPC tumors retain characteristics of prostate adenocarcinoma, some may become poorly differentiated losing their luminal structure and/or acquiring characteristics of classical small cell carcinoma (Epstein *et al.* 2014). Small cell carcinoma is considered a poorly differentiated neuroendocrine carcinoma and can look and act similar to small cell neuroendocrine carcinomas arising in other anatomic sites (e.g. small cell lung cancer (SCLC)). While immunohistochemistry (IHC) is not required for the diagnosis of NEPC, IHC is often performed clinically as cells typically express classical neuroendocrine markers such as chromogranin, synaptophysin, INSM1, NSE, CD56 (Epstein *et al.* 2014). It is important to note that expression of these neuroendocrine markers is sometimes observed in poorly differentiated/high-grade adenocarcinomas. Large cell carcinoma variants or tumors with mixed features of small cell or large cell carcinoma admixed with classical prostate adenocarcinoma are also observed in later stages of prostate cancer. The term 'neuroendocrine prostate cancer (NEPC)' has been used to broadly encompass prostate cancers that have neuroendocrine features based on morphology and IHC staining for neuroendocrine markers. While NEPC tumors often lack expression of the androgen receptor (AR) and downstream AR-regulated targets (such as prostate-specific antigen (PSA)), this is not universal, reflecting the spectrum and continuum of lineage plasticity (Beltran *et al.* 2019). Of note, a subset of CRPC tumors lack morphologic features and IHC markers of NEPC and also lack AR expression and have thus been termed 'double negative prostate cancer' (Bluemn *et al.* 2017). Whether this represents a distinct entity or a state within the continuum of lineage plasticity toward NEPC is not well established clinically. Other acquired alternative lineage phenotypes such as squamous and gastrointestinal programs have also been observed in later stage CRPC (Shukla *et al.* 2017, Labrecque *et al.* 2021), suggesting potentially differential trajectories within the lineage plasticity process. Clinically, patients with NEPC typically develop progression in the setting of low or non-rising serum PSA levels (but not always) and may have elevated serum neuroendocrine markers (such as chromogranin, NSE) or carcinoembryonic antigen (CEA) levels and/or

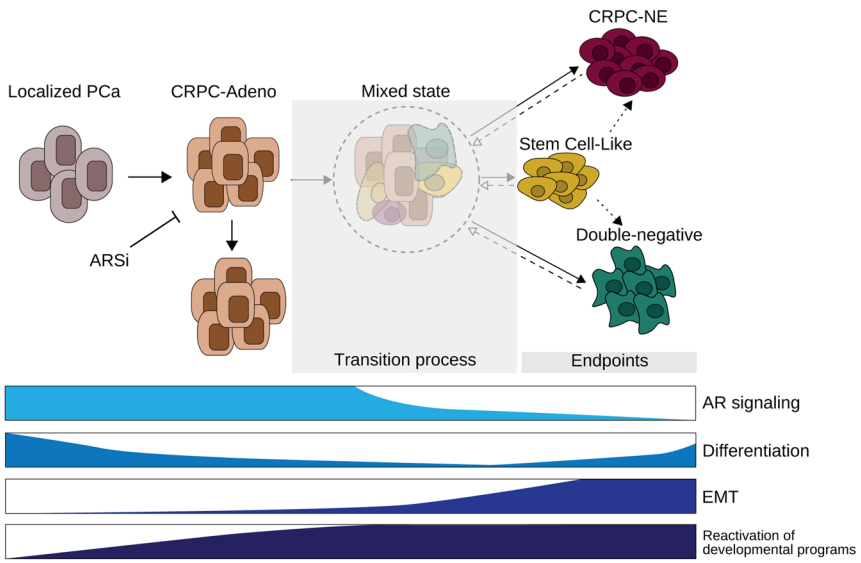

Figure 1
Schematic of the lineage plasticity process in prostate cancer.

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develop visceral or atypical sites of metastases (e.g. lytic bone, parenchymal brain) (Conteduca *et al.* 2019). While these clinical characteristics are not specific for NEPC, aggressive clinical features have been used as inclusion criteria for clinical trial enrolment (Aparicio *et al.* 2013, Corn *et al.* 2019). Specifically, the aggressive variant prostate cancer (AVPC) criteria have been used within platinum chemotherapy trials in CRPC and include the presence of exclusive visceral metastases, low PSA and bulky disease, high lactose dehydrogenase levels, elevated CEA, lytic bone metastases, and/or NEPC histology (Aparicio *et al.* 2013).

While most NEPC tumors are recognized in later stages after patients have had prior therapies for metastatic CRPC, they can also arise *de novo*, either as pure small cell carcinoma or mixed with high-grade adenocarcinoma. Both treatment-emergent and 'de novo' NEPC share common genomic aberrations as prostate adenocarcinoma (e.g. with the prostate cancer-specific *TMPRSS2-ERG* fusion present in approximately 40–50%) (Beltran *et al.* 2011, Chedgy *et al.* 2018), supporting their common cellular origin, but they are enriched with certain acquired alterations including loss of *RB1* and *TP53*–alterations universally present in other poorly differentiated neuroendocrine carcinomas such as SCLC and acquired in EGFR-mutated lung adenocarcinomas that transform to SCLC (George *et al.* 2015, Niederst *et al.* 2015, Beltran *et al.* 2016*b*, Park *et al.* 2018). The presence of *RB1* loss or combined tumor suppressor losses (*RB1, TP53, PTEN*) in men with CRPC even without histologic evidence of NEPC has been associated with aggressive disease and poor prognosis (Aparicio *et al.* 2016, Abida *et al.* 2019). Epigenetic alterations, including changes in DNA methylation, chromatin accessibility, SWI/SNF, and histone marks are distinguishing features of NEPC, suggesting a key role of epigenetics in driving histologic transformation (or *trans-differentiation*) from prostate adenocarcinoma to NEPC (Beltran *et al.* 2016*b*, Dardenne *et al.* 2016, Cyrta *et al.* 2020, Baca *et al.* 2021). Activation and coordination of lineage determining transcription factors (e.g. ASCL1, BRN2, ONECUT2, MYCN, FOXA1) (Dardenne *et al.* 2016, Lee *et al.* 2016, Bishop *et al.* 2017, Rotinen *et al.* 2018, Guo *et al.* 2019, Baca *et al.* 2021), pluripotency factors (e.g. SOX2) (Bishop *et al.* 2017, Mu *et al.* 2017), and homeobox genes (eg. HOXB5, HOXB6) (He *et al.* 2021), as well as dysregulation of prostate cancer genes in the context of AR therapy (eg. ERG, SPINK1) (Mounir *et al.* 2015, Tiwari *et al.* 2020), and/or downregulation of other transcription factors (e.g. REST) (Lapuk *et al.* 2012, Zhang *et al.* 2015) further drive transcriptional changes and associated lineage reprogramming (He *et al.* 2021). This lineage reprogramming may be mediated by an intermediary, de-differentiated 'stem like' state before cells differentiate toward a neuronal developmental/neuroendocrine-like phenotype with loss of AR dependence (Fig. 1). Based on the study of metastatic tumors, we developed a 70-gene signature (NEPC score) that distinguishes NEPC from prostate adenocarcinoma (Beltran *et al.* 2016*b*). Interestingly, this NEPC score has been found to be inversely correlated with AR signaling not only in metastatic CRPC (Abida *et al.* 2019) but also in primary untreated tumors (Mahal *et al.* 2018).

With significant advances in our understanding of the biological underpinnings of lineage plasticity and the mechanisms that drive the development of NEPC, there is a growing need for biomarkers to (1) more accurately diagnose NEPC in the clinic and (2) potentially predict NEPC transformation before it develops or identifies patients in the process of lineage plasticity while progressing on standard therapies for early intervention strategies. This has important therapeutic implications. For instance, if the target of interest is expressed only when neuroendocrine lineage is present, then an accurate biomarker to identify NEPC and/or the target would be needed. If the goal of therapy is to prevent or reverse lineage plasticity (for instance, through epigenetic modulation), then a biomarker to identify tumors without neuroendocrine differentiation would be ideal, and co-targeting or alternating strategies (targeting AR and non-AR pathways) may be rationally developed.

Relationship between target and therapy

Patients with histologically confirmed small cell NEPC or CRPC with aggressive variant clinical features (AVPC) are often treated with systemic therapy regimens used for SCLC, based on clinical, pathologic, and molecular similarities between NEPC and SCLC (Berchuck *et al.* 2021). Similar to SCLC, they tend to be initially responsive to platinum-based chemotherapy with objective response rates of 50–60% (Sella *et al.* 2000, Papandreou *et al.* 2002). The combination of carboplatin with the taxane chemotherapy cabazitaxel is now supported by the National Comprehensive Cancer Network (NCCN) guidelines as an option for patients with aggressive variant clinical features or unfavorable genomics (loss of function alterations involving at least two of *PTEN*, *TP53*, and *RB1*). In a phase II study, 113 men meeting AVPC clinical criteria were treated with carboplatin plus docetaxel followed by cisplatin plus etoposide at progression (Aparicio *et al.* 2013). The median progression-free survival (PFS) with

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carboplatin plus docetaxel was 5.1 months, and the median overall survival was 16.0 months (95% CI 13.6–19.0) (Aparicio *et al.* 2013). In a follow-up randomized phase II study (Corn *et al.* 2019), 160 men with metastatic CRPC, stratified by the presence of AVPC features, were randomized to cabazitaxel vs cabazitaxel plus carboplatin. There was a more substantial improvement in PFS favoring combination chemotherapy (cabazitaxel plus carboplatin) in men with AVPC (HR 0.58 (95% CI 0.37–0.89) compared with those without AVPC (HR 0.74 (95% CI 0.46–1.21)). The mechanisms of response of NEPC and AVPC to this regimen may also be influenced by underlying tumor suppressor loss and/or DNA repair aberrations. The combination of cabazitaxel with carboplatin has a particular rationale in NEPC, given the activity of cabazitaxel in CRPC and mixed tumor histologies (both adenocarcinoma and NEPC elements) frequently observed within the spectrum of NEPC (Epstein *et al.* 2014).

For those patients with pure small cell carcinoma, SCLC regimens such as carboplatin-etoposide with or without the anti-PDL1 immune checkpoint inhibitor atezolizumab (based on the IMpower133 trial (Horn *et al.* 2018)) may also be considered. Docetaxel with carboplatin is also a reasonable option for patients who have not previously received docetaxel, given the frequent mixed CRPC/NEPC features. A major clinical challenge is in what to do next after platinum-based chemotherapy. There are limited data in the second line and beyond settings. For mixed tumors with both adenocarcinoma and NEPC elements, the choice of therapy depends on the dominant histology and clinical context. Next line SCLC regimens may be considered for those with small cell NEPC; these include lurbinectedin, topotecan, pembrolizumab, ipilimumab/nivolumab. Given limited data, we would encourage participation in clinical trials. Further, ADT should be considered in both *de novo* and treatment-related NEPC cases (even small cell carcinomas) given tumor heterogeneity.

Emerging targets for lineage plasticity and NEPC have been discovered through combined clinical and preclinical studies (Fig. 2), and several trials are now underway. Several of these targets are shared with SCLC, such as DLL3, AURKA, EZH2, and DNA repair pathways. Understanding the relationship between target expression and pathway activation with therapeutic responses to these agents will be critical for patient selection, especially since these features may not be present at the time of the patient's initial diagnosis. NEPC tumors express cell surface markers that may be exploited therapeutically. CEACAM5 (carcinoembryonic antigen-related cell adhesion molecule 5) and DLL3 (delta-like ligand 3) are two examples of cell surface proteins also present in other cancers such as colon cancer and SCLC, respectively (Thompson *et al.* 1991, Saunders *et al.* 2015). Lee *et al.* (2018) evaluated cell surface profiles using gene expression data and validated CEACAM5 as a marker enriched in NEPC and a promising target for cell-based immunotherapy. As a proof of concept, they developed an engineered chimeric antigen receptor T cells targeting CEACAM5, which induced antigen-specific cytotoxicity in NEPC models (Lee *et al.* 2018). Further clinical investigation targeting CEACAM5 is planned for men with NEPC. We identified DLL3 as another cell surface protein, a notch inhibitory ligand, highly expressed in the

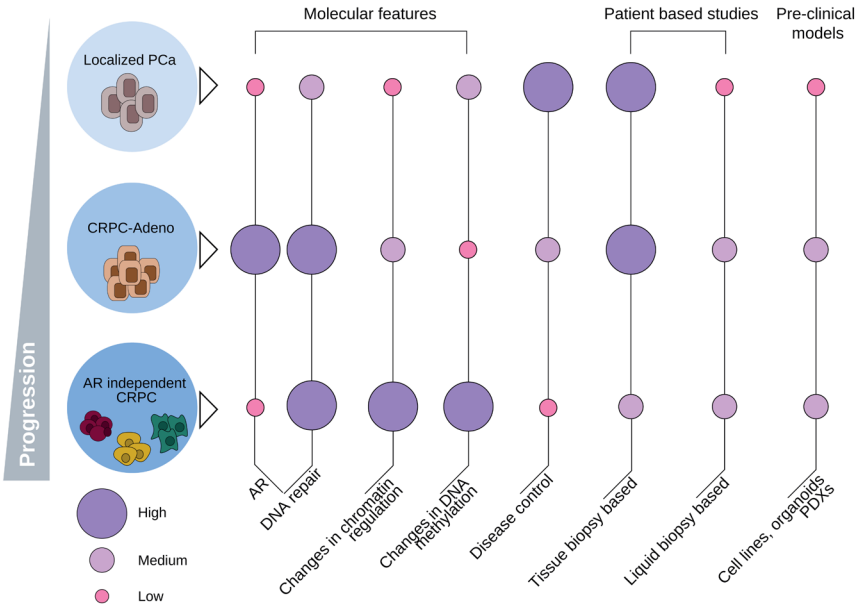

Figure 2
Representation of relative abundance of features and patient-based studies and models along the continuum from localized prostate cancer (PCa) to castration-resistant adenocarcinoma (CRPC-Adeno) to androgen receptor (AR)-independent CRPC. The schematic includes the frequencies of relevant genomic and epigenomic aberrations associated with neuroendocrine prostate cancer and the relative number of patient-based studies and models that exist to study each of these disease states, evidencing the yet modest availability of tissue-based and liquid biopsy-based studies in the setting of AR independent CRPC, where disease control is especially limited. AR, androgen receptor; CRPC, castration-resistant prostate cancer.

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majority of NEPC (76.6%) as well as a subset of aggressive CRPC tumors (12.5%), and not expressed in benign tissues (Puca *et al.* 2019). DLL3 is being investigated as a therapeutic target for both NEPC and SCLC. The anti-DLL3 × CD3 Bi-specific T cell Engager AMG757 (Amgen) and the DLL3-targeted tri-specific T cell activating construct HPN328 (Harpoon) are in early phase clinical trials (NCT04471727, NCT04702737).

Other immune targeted approaches are also being investigated in NEPC. Though immune checkpoint inhibitors are commonly used in SCLC and sometimes extrapolated to treat small cell NEPC, there have been limited data reported to date for this subgroup of prostate cancers. In a recent study by Brady *et al.* (2021), digital spatial profiling revealed that the majority of metastatic CRPC and NEPC tumors are devoid of significant inflammatory infiltrates and lack PD1, PD-L1, and CTLA4. Consistent with this, Brown *et al.* (GU ASCO 2021) recently reported results of 19 patients with NEPC or aggressive variant clinical features treated with the PD-L1 inhibitor avelumab in a single-arm phase 2 study (NCT03179410). Radiographic response rate was 6.7%. One complete response was observed in a patient that also harbored tumor microsatellite instability and MSH2 mutation. Median radiographic progression-free survival was 1.8 months (95% CI 1.6–2.0) and median time on therapy was 56 days (range 28–356). Median overall survival was 7.4 months (85% CI 2.8–12.5). Given this limited single-agent activity, combination immunotherapy is now being tested. Ongoing clinical trials include a phase 2 basket study of ipilimumab plus nivolumab for rare GU tumors with a small cell carcinoma arm (NCT03333616), a phase 1/2 study of pembrolizumab plus BXCL701, an oral innate immunity activator (NCT03910660), and a phase 1 study of pembrolizumab in combination with platinum-based chemotherapy (NCT03582475).

NEPC tumors express higher levels of cell cycle genes including aurora kinase A (AURKA), aurora kinase B (AURKB), and polo kinase (PLK1) (Beltran *et al.* 2011). Aurora-A has also been shown to stabilize N-myc during NEPC progression (as also observed in neuroblastoma, another neuroendocrine tumor (Otto *et al.* 2009)). Expression of Aurora A has also been linked mechanistically with TP53 mutation via expression of *miR-25* leading to reduced levels of FBXW7 which encodes an E3 ubiquitin ligase that regulates Aurora A (Li *et al.* 2015). Targeting aurora kinase in the context of underlying *RB1* loss (present in the majority of NEPC) is synthetically lethal (Gong *et al.* 2019, Oser *et al.* 2019), providing additional rationale. The Aurora kinase A inhibitor alisertib was tested in a phase 2 clinical trial for patients with clinical or pathologic features of NEPC (Beltran *et al.* 2018). Though this study did not meet its primary endpoint of PFS in a biomarker-unselected population, exceptional responders were observed. Further investigation, potentially in the context of *N*-myc gain or RB1 loss, may be warranted. Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase that also cooperates with *N*-myc to drive neuroendocrine differentiation, and chemical and pharmacologic inhibition of ALK has shown activity in preclinical models of both NEPC and *N*-myc-driven neuroblastoma; these data point to additional potential shared vulnerabilities between NEPC and other neuroendocrine tumors that may be exploited clinically (Unno *et al.* 2021). RET is another kinase identified as preferentially activated in NEPC with potential therapeutic implications (Drake *et al.* 2013). Although the phase 3 trial of cabozantinib (which targets RET as well as other kinases such as MET, VEGFR2, FLT3, c-KIT) was negative for overall survival in metastatic CRPC (Smith *et al.* 2016), it is currently being evaluated in a biomarker-selected subgroup of CRPC (NCT04631744) as well as in combination with atezolizumab (NCT04446117).

Targeting DNA repair is an area of interest in NEPC, given clinical responses to platinum chemotherapy and similarities with SCLC. The frequency of genomic aberrations involving DNA repair pathways is similar in treatment-related NEPC as other CPRC tumors (approximately 20% harboring pathogenic mutations in homologous recombination genes), and potentially more common in *de novo* cases (Beltran *et al.* 2016*b*, Chedgy *et al.* 2018). Upregulation of DNA repair genes in NEPC has also been associated with activation of PARP1/2 by *N*-Myc (Zhang *et al.* 2018, Liu *et al.* 2019). While poly (ADP-ribose) polymerase (PARP) inhibitors are currently indicated for specific genomic alterations linking with the Food and Drug Administration (FDA) label in CRPC, their use in additional contexts is not yet established. A phase 2 study of induction chemotherapy with carboplatin plus cabazitaxel followed by maintenance PARP inhibitor therapy with olaparib in patients with aggressive variant clinical features including NEPC is ongoing (NCT03263650). The combination of PARP inhibitor and ataxia telangiectasia and rad3-related (ATR) inhibitor therapy has also shown preclinical activity in CRPC tumors with combined TP53 and RB1 loss, reflecting a potential targetable vulnerability resulting from replication stress (Nyquist *et al.* 2020). In SCLC, combined inhibition of ATR (bertosertib) and topoisomerase (topotecan) is also synergistic; an overall response rate of 36% was seen with this combination including durable regressions in patients with platinum-

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resistant disease (Thomas *et al.* 2021). SCLC tumors with high neuroendocrine differentiation and enhanced replication stress were more likely to respond. These data suggest that replication stress may also be a potential therapeutic vulnerability in NEPC.

Though lineage plasticity may be facilitated by underlying genomic aberrations, such as loss of RB1 and TP53, the most striking differences between NEPC and prostate adenocarcinoma are epigenetic. Recent DNA methylation, ChIPseq, and ATACseq studies support transcriptional reprogramming mediated by epigenetic regulators and driven by key lineage determining transcription factors (TFs). EZH2 is an epigenetic regulator, part of the polycomb repressive complex 2 (PRC2), involved in histone methylation (H3K27me3) and gene repression. EZH2 is overexpressed in CRPC compared with primary tumors, and even more so in NEPC (Beltran *et al.* 2016*b*, Ku *et al.* 2017, Puca *et al.* 2018). There is a suggestion based on preclinical studies that targeting EZH2 can reverse plasticity in the setting of underlying tumor suppressor loss or *N*-myc gain, re-sensitizing tumors to enzalutamide (Dardenne *et al.* 2016, Kleb *et al.* 2016, Ku *et al.* 2017). EZH2 can also activate the AR in the setting of AR-driven disease (Xu *et al.* 2012). Clinical trials investigating the combination of EZH2 inhibitor therapy with potent AR inhibition are underway for late-stage CRPC (NCT03480646, NCT03460977, NCT04179864). Ongoing correlative studies may elucidate molecular subsets within responders in these trials.

Targeting other epigenetic regulators, such as LSD1, BET, or DNA methyltransferase, are also under investigation in advanced CRPC and may have a rationale in NEPC (Conteduca 2021). Current ongoing trials of epigenetic drugs are also without patient selection for NEPC. Similarly, correlative studies may elucidate molecular mechanisms of response in these trials. This will require not only studying mediators of anti-tumor activity but also potential changes in tumor lineage programs that occur on therapy. DNA methylation changes, in particular, are distinguishing features of NEPC, detectable in both metastatic tumors and cell-free DNA (plasma) of patients (Beltran *et al.* 2016*a*, 2020, Zhao *et al.* 2020). Integration of DNA methylation with transcriptomic data suggests epigenetic regulation of cell–cell adhesion, developmental, and stem-like pathways. Targeting DNA methyltransferases and/or other upstream mediators that regulate these processes are approaches under investigation. Of note, downregulation of protein kinase C (PKC) λ/ι results in the upregulation of serine biosynthesis and increases intracellular S-adenosyl methionine levels in NEPC, pointing to a potential metabolic regulation of DNA methylation during lineage plasticity (Reina-Campos *et al.* 2019). Additionally, the downstream TFs that mediate transcriptional changes associated with lineage reprogramming, such as BRN2, ASCL1, FOXA1, ONECUT2, MYCN, and others may also serve as potential therapeutic targets though TFs are much more challenging to target directly. Understanding the mechanisms and timing of epigenetic events and their cooperation in promoting and/or sustaining plasticity will be important for the development of rational biomarkers and combination strategies. If AR can indeed be re-activated by targeting epigenetic alterations, then combination or bipolar/alternating strategies may be tested.

Emerging biomarkers

Several tissue-based studies and preclinical studies have identified genomic, transcriptomic, and epigenetic differences between castration-resistant prostate adenocarcinoma and NEPC (Beltran *et al.* 2019). While these studies have fueled mechanistic studies and validation of new targets, in practice obtaining metastatic biopsies for extensive molecular analyses is a major clinical challenge. Biopsies, when performed to evaluate for NEPC, are often small and prioritized clinically to confirm the diagnosis (tumor morphology, IHC) and for targeted genomic assays to identify targets for FDA-approved therapies (e.g. olaparib, rucaparib, pembrolizumab). IHC staining for classical neuroendocrine markers is commonly performed clinically and may potentially be improved upon with other NEPC-associated markers that can be measured *in situ* (e.g. IHC for RB1, DLL3, ASCL1, SWI/SNF complex members). The presence of combined tumor suppressor loss (*RB1*, *TP53*, *PTEN*) is often detected on targeted genomic assays in patients with NEPC as well as those with aggressive variant clinical features and may help identify patients for more aggressive systemic therapy regimens such as platinum-based chemotherapy, as recently endorsed by the NCCN (Aparicio *et al.* 2016). The timing of their acquisition and evolution of these tumor suppressor losses, and the contribution of other co-occurring genomic and epigenomic DNA alterations, have not been extensively studied. To this end, we evaluated molecular changes in CRPC and NEPC in the context of a few specific drug targets (Fig. 3) illustrating how DNA-based changes (e.g. tumor suppressor loss, DNA methylation) might correlate with targets/pathways in CRPC or NEPC that are non-genomic based.

Despite the increased recognition of patients with CRPC that progress toward NEPC, the total number of profiled

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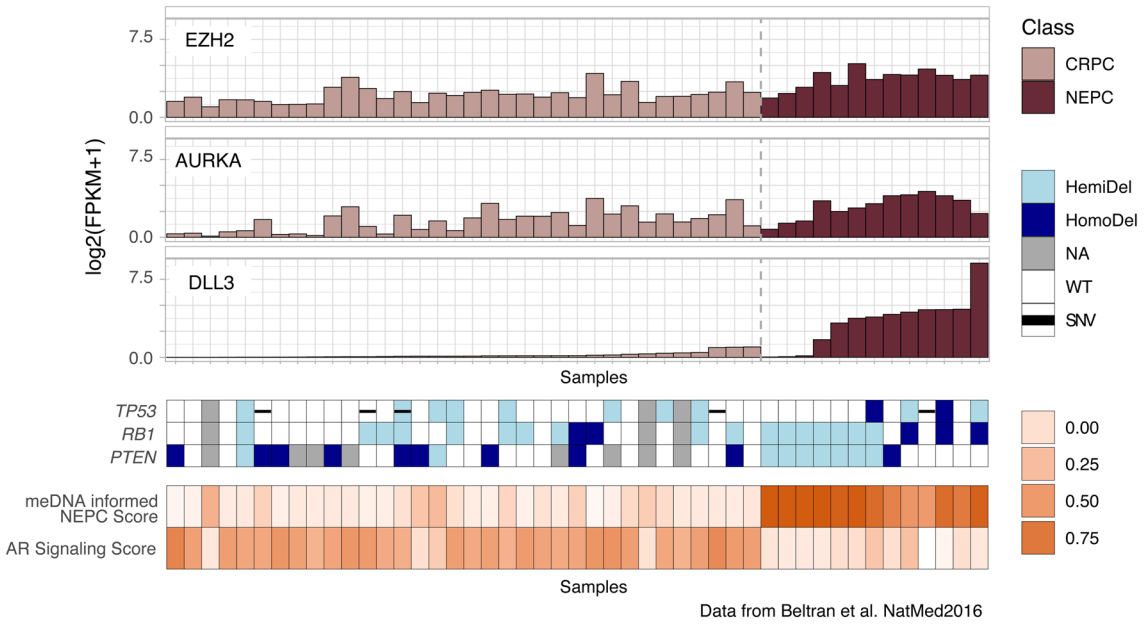

Figure 3
Gene expression of specific targets (EZH2, AURKA, DLL3) in CRPC and NEPC metastatic tissue biopsies, annotated by genomic tumor suppressor loss, NEPC-associated features informed by DNA methylation changes, and AR signaling. Epigenetic and genomic signals might be indicative of non-genomic-based NEPC relevant targets/pathways. EZH2, enhancer of zeste 2; DLL3, delta-like ligand 3; CRPC, castration-resistant prostate cancer; NEPC, neuroendocrine prostate cancer; AR, androgen receptor.

patient tumors in the published literature remains low and there are differences in the diagnostic criteria, platforms, and analytics used. One challenge lies in the need for invasive biopsies. Evaluation of primary tumors suggests that most NEPC features (e.g. histology, RB1 loss, DNA methylation changes) are not typically detected early; however, there may be transcriptomic changes associated with AR independence early on (Mahal *et al.* 2018, Spratt *et al.* 2019). Whether these transcriptomic changes predict the future development of NEPC and/or therapy response in earlier disease settings (e.g. AR therapy vs chemotherapy) is yet to be reported. Given that most of the targetable alterations identified in NEPC appear to be acquired during therapy resistance, the development of non-invasive biomarkers (e.g. liquid biopsies, molecular imaging) to detect molecular changes associated with NEPC is a promising approach. Serial assessments allow for the charting of tumor dynamics over time and for specifically analyzing treatment-related changes and target expression. Furthermore, single-site metastatic biopsies do not always reflect the pathologic heterogeneity that may be seen within an individual patient; therefore, non-invasive biomarkers have an additional advantage in more accurately reflecting composite tumor burden and molecular intra-patient heterogeneity.

Especially in the setting of heterogeneous diseases such as metastatic prostate cancer, plasma cell-free DNA (cfDNA) studies have provided valuable observations, both at the genomic and epigenomic level. Relevant to the characterization of NEPC, we analyzed biopsy-confirmed castration-resistant adenocarcinoma and NEPC tumors along with matched cfDNA by whole-exome sequencing and whole-genome bisulfite sequencing and found that (1) cell-free DNA genomics recapitulates the relative contribution of key drivers (including loss of TP53, RB1, PTEN, amplification of MYC, MYCN, and AR), (2) serial sampling can track the emergence and clonal dominance of certain sub-clonal alterations, (3) the level of intra-patient genomic heterogeneity is higher in castration-resistant adenocarcinoma compared with NEPC, and (4) DNA methylation of cfDNA resembles tissue-based data and can identify NEPC-specific features (Beltran *et al.* 2020). Further, through serial sample analyses, we demonstrated that NEPC-associated DNA changes could be detected in the circulation prior to clinical evidence of NEPC. Key to this initial study was the availability of metastatic tissue biopsies that allowed for the assessment of inter and intrapatient similarities on a metastatic site basis and across sites, leading to the observation that NEPC-associated alterations are generally conserved across sites, and, therefore, the data from cfDNA were not driven by the sites of metastases. There are several commercial platforms available for cell-free DNA analysis. While targeted cfDNA

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platforms are commonly used clinically in metastatic CRPC to evaluate for actionable genomic alterations, cfDNA methylation platforms such as GRAIL and others have mostly been geared toward cancer detection and minimal residual disease monitoring. Additional work is needed to leverage such technologies for the detection of specific cancer resistance phenotypes such as NEPC.

While the cfDNA sequencing is informative of genomic burden, the mutational load, the presence of specific somatic DNA and DNA methylation events, the noninvasive assessment of transcriptional profiles and expression of key targets are more challenging. Ulz and colleagues recently proposed a method for assessing transcription factor activity via cfDNA (Ulz *et al.* 2019). Specifically, through whole-genome sequencing of cfDNA and nucleosome footprint analysis, they inferred transcription factor accessibility based on DNA fragmentation patterns. The authors showed the ability to distinguish NEPC from prostate adenocarcinoma based on differential accessibility of the binding sites of AR, REST, HOXB13, NKX3-1, and GRHL2. Potentially combining novel approaches with cfDNA methylation, cfDNA ChIPseq, or other technologies will provide a more comprehensive readout of the epigenetic landscape of NEPC with the ability to track dynamic and functional changes while on therapy.

Circulating tumor cells (CTCs) hold promise for not only detecting tumor burden (e.g. quantifying CTC count) but can also test morphologic differences between NEPC and adenocarcinoma tumor cells, and other drug targets and resistance mechanisms through mRNA or protein analyses. Using the Epic CTC platform, we identified low or absent AR expression, low cytokeratin expression, smaller morphology, and cell clusters more frequently in CTCs from men with NEPC compared to those with castration-resistant prostate adenocarcinoma (Beltran *et al.* 2016*a*). In another study of patients with AVPC, the presence of copy number alterations involving at least two tumor suppressor genes and genomic instability in DNA derived from single CTCs was associated with poor survival (Malihi *et al.* 2020). Overall, CTC heterogeneity has been associated with poor prognosis in patients with CRPC treated with AR-targeted therapies (Scher *et al.* 2017). In the multicenter PROPHECY study, CTCs were evaluated by the Epic platform in 107 men prior to receiving abiraterone or enzalutamide (Brown *et al.* 2021). Of these, 8.7% had NEPC features defined by cell morphology and size, and their presence along with chromosomal instability in CTCs was associated with poor prognosis. In addition to providing prognostic value, CTCs are also capable of detecting other tumor features such as expression of AR-V7, SLFN11, and DLL3 (Puca *et al.* 2019, Armstrong *et al.* 2020, Conteduca *et al.* 2020, McKay *et al.* 2021).

Extracellular vesicles (EVs) derived from metastatic tumor cells can also be detected in the circulation in men with CRPC, including those that are CTC-negative, and may carry tumor-associated proteins that can be quantified and tracked serially (Gerdtsson *et al.* 2021). The role of EVs as biomarkers to detect lineage plasticity is an area of active investigation (Bhagirath *et al.* 2021). There are also emerging data regarding the ability of EVs to reprogram cells and they may play a functional role in driving lineage plasticity. For instance, EVs from integrin αVβ3 expressing prostate cancer cells are capable of stimulating tumor growth and driving neuroendocrine differentiation in tumor models (Quaglia *et al.* 2020).

Molecular imaging is another emerging non-invasive biomarker in CRPC. Recent studies have been focused on detecting prostate-cancer specific antigen (PSMA) expression in tumors through PET – computerized tomography (CT) imaging, as well as targeting PSMA lesions via radionuclide therapy (e.g. Lu-PSMA-617) or other approaches (Miyahira *et al.* 2020). There is a subset of CRPC that express lower levels of PSMA or lose PSMA expression in later stages of the disease, which can also be detected by PSMA PET-CT and metabolically-avid lesions confirmed with concurrent fluorodeoxyglucose (FDG) PET-CT (Thang *et al.* 2019). PSMA negativity or PSMA/FDG discordance on PET imaging has been associated with aggressive disease and poor prognosis (Thang *et al.* 2019). When tumors lose AR expression (as seen in NEPC), PSMA expression may also be lost (Bakht *et al.* 2018). Whether PSMA/FDG imaging can be incorporated to improve the detection of NEPC is an area of active investigation. Efforts to detect NEPC via somatostatin receptor expression using Ga-68-DOTATOC PET/CT have shown mixed results, with overall lower expression of somatostatins in NEPC compared with well-differentiated neuroendocrine tumors of the gastrointestinal tract or lung (Hope *et al.* 2015, Iravani *et al.* 2021). Other PET tracers such as those evaluating DLL3 (Sharma *et al.* 2017) are also under investigation.

Conclusions

In summary, NEPC is an aggressive variant of prostate cancer that most commonly develops in later stages of prostate cancer progression as a mechanism of resistance. Platinum-based chemotherapy such as carboplatin plus cabazitaxel has activity and is endorsed by NCCN guidelines, but the second line and beyond settings are

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major clinical challenges. While there may be certain molecular features present at initial diagnosis that may contribute toward future development of lineage plasticity or AR independence, most NEPC alterations are acquired later. Metastatic biopsy studies and recent preclinical research have identified genes and pathways that coordinate together to turn 'off' the AR-driven luminal prostate program and turn 'on' the neuroendocrine lineage. Plasticity is facilitated by genomic aberrations (e.g. combined RB1, TP53 loss), and NEPC is characterized by distinct epigenetic changes. Trials targeting emerging genes/pathways in NEPC will require careful patient selection. Metastatic biopsies are invasive and challenged by pathologic heterogeneity even within individual patients. Incorporating non-invasive biomarkers to detect molecular features of NEPC (e.g. liquid biopsies, molecular imaging) into ongoing clinical studies across the prostate cancer continuum is essential. This will help track the lineage process and facilitate prioritization, and the validation studies needed for their incorporation into future clinical trials aimed at targeting NEPC and/or reversing plasticity.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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