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Bad neighbours: hypoxia and genomic instability in prostate cancer

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ABSTRACT:
Prostate cancer (PCa) is a clinically heterogeneous disease and has poor patient outcome when tumours progress to castration-resistant and metastatic states. Understanding the mechanistic basis for transition to late stage aggressive disease is vital for both assigning patient risk status in the localised setting and also identifying novel treatment strategies to prevent progression. Subregions of intratumoral hypoxia are found in all solid tumours and are associated with many biologic drivers of tumour progression. Crucially, more recent findings show the co-presence of hypoxia and genomic instability can confer a uniquely adverse prognosis in localised PCa patients. In-depth informatic and functional studies suggest a role for hypoxia in co-operating with oncogenic drivers (e.g., loss of PTEN) and suppressing DNA repair capacity to alter clonal evolution due to an aggressive mutator phenotype. More specifically, hypoxic suppression of homologous recombination represents a “contextual lethal” vulnerability in hypoxic prostate tumours which could extend the application of existing DNA repair targeting agents such as poly-ADP ribose polymerase inhibitors. Further investigation is now required to assess this relationship on the background of existing genomic alterations relevant to PCa, and also characterise the role of hypoxia in driving early metastatic spread. On this basis, PCa patients with hypoxic tumours can be better stratified into risk categories and treated with appropriate therapies to prevent progression.

INTRODUCTION
Prostate cancer (PCa) is one of the most prevalent cancers in males. In the UK alone, there are currently 47,500 new cases and 11,500 deaths per year.1 As with many cancers, patient prognosis commonly correlates with the stage of disease at diagnosis. In PCa, this is largely driven by tumour dependency on steroidal androgen hormones and concomitant response to androgen deprivation therapy (ADT).2,3 The disease landscape therefore spans indolent localised disease, potentially curable with ADT, radiotherapy or radical prostatectomy, to aggressive and incurable metastatic castration resistant PCa (mCRPC)4 (Figure 1). Correspondingly, the respective 5 year survival rate falls from near 100% in early stage patients to roughly 30% in mCRPC patients.9 Therefore, although the majority of patients present with localised disease at diagnosis, there are two clear areas within the field which require urgent investigation. The first is to improve therapy for mCRPC patients and the second to identify patients who have localised disease but are at high risk for occult metastasis and subsequent progression, thus requiring treatment intensification.5 On top of this, a mechanistic understanding of why these patients progress so rapidly must be established to facilitate early detection and the development of novel treatment strategies.

Besides the widely recognised risk factors of advanced age and ethnicity, key molecular processes such as inflammation, oxidative stress and DNA damage have been implicated in driving genomic alterations and propensity for carcinogenesis.11 Taken together, these processes suggest a key role of the tumour microenvironment (TME) in promoting PCa. As tumours grow and cancer cells disseminate further from an increasingly chaotic vasculature, the TME can be characterised by subregions of nutrient deprivation, low pH and low tissue oxygenation, termed hypoxia.4 This review will focus on the mechanistic and prognostic role of hypoxia across PCa in the non-metastatic and metastatic states (Figure 1). It will particularly review the biochemical and cellular interplay...
between hypoxia with DNA repair competency as a driver of genomic instability.

**HYPOXIA AND PROSTATE TUMOUR PROGRESSION**

In most tumours, a state of hypoxia can generally be classified as acute or chronic, dependent on the nature of the decrease in tissue oxygenation. Acute or cycling hypoxia arises where perfusion is temporarily limited, often due to abnormal tumour vasculature. Alternatively, chronic hypoxia arises when proliferating cells become progressively distant from the nearest blood vessel, thus increasing diffusion distance and reducing relative oxygen and nutrient supply for a prolonged time. These cells often remain hypoxic until cell death, although under certain scenarios such as treatment induced cell kill of cells close to blood vessels, distal hypoxic cells may become re-oxygenated and replenish the tumour, leading to disease recurrence. Both the direct quantitative measurement of hypoxia using fine needle piezo-electrodes or uptake of bioreductive compounds with subsequent biopsy staining and development of hypoxic gene signatures is consistent with heterogeneous intratumoral hypoxic fractions between PCa patients. As a consequence, the prostate is an organ now known to have relatively low oxygenation compared to other tissues and numerous studies have implicated hypoxia as a key driver in carcinogenesis for prostate and other types of cancer.

Adaptation of cancer cells to hypoxia has also been shown to promote aggressiveness by further driving genomic instability, resistance to therapy and likelihood of metastasis, thus leading to poor patient prognosis. Bioinformatic studies have linked hypoxia with increased mutational load across cancers, irrespective of the underlying mutational class. The proportion of mutations attributed to several mutational signatures of unknown aetiology are directly associated with the level of hypoxia, suggesting underlying mutational processes for these signatures. At the gene level, driver mutations in TP53, MYC and PTEN are enriched in tumours with high hypoxia, and mutations in PTEN interact with hypoxia to direct the evolutionary trajectory of tumours. The intersection of hypoxia and increasing genetic alteration in PCa starts to explain the observation that hypoxia selects for androgen independence and neuroendocrine PCA differentiation (Figure 1), associates with PTEN loss, increased chromothripsis and elevated percentage genome alteration (PGA), and was prognostic in a cohort of 247 localised PCa patients. These studies confirm hypoxia is not simply a consequence of the mismatch between high tumour oxygen consumption and limited delivery, but additionally plays a leading role in oncogenesis and the clinical course of disease.

This vital role of hypoxia raises the question as to whether hypoxia acts to compound existing genomic aberrations. With this in mind, it has been suggested that composite methods of characterising genomic instability, aggressive subpathologies and hypoxia in localised PCa patients is more prognostic than classical methods of PCa stratification such as histological-based Gleason Score (GS) and measurement of prostate-specific antigen (PSA) in the serum, alone. Indeed for both radiotherapy and radical prostatectomy treated cohorts, hypoxia and genomic instability had an independent and additive effect on biochemical relapse. More in-depth genome sequencing studies show hypoxia and genomic instability track across 50% of all cancers and targeted approaches toward this interaction, for instance...
example poly-ADP ribose polymerase (PARP) inhibition, should now be considered.21

THE BIOCHEMISTRY OF HYPOXIA: IMPLICATIONS FOR PROSTATE CANCER

In response to acute or chronic hypoxia, cells regulate distinct processes to guard against cell death.13 Major players in these adaptive responses are the heterodimeric proteins hypoxia inducible factor (HIF) 1 and 2.28 Under hypoxic conditions, HIF1α and HIF2α become stabilised, dimerise with HIF1β/HIF2β respectively and regulate target gene expression (Figure 2). HIF1α and HIF2α have hundreds of target genes and hence the downstream biology is exceptionally complex, with various key processes in cancer progression implicated to be HIF1-dependent28 (Table 1). These include vascular endothelial growth factor A (VEGF-A) mediated angiogenesis,36 as well as upregulation of the transmembrane glucose transporter GLUT1 and intracellular pH regulator carbonic anhydrase IX (CAIX) to facilitate a metabolic switch to aerobic glycolysis.20,41 Other HIF1-dependent pathways include signalling tumour cell invasion,8 immune dampening,30 formation of a tumour stem cell protective niche,31 and signalling to induce pre-metastatic niche formation in secondary organs.9 HIF1α, VEGF-A and GLUT1 have all been associated with reduced time to biochemical failure in PCa, while a more recent meta-analysis also suggested HIF-2α is a negative prognostic factor for metastasis free survival.29,33,38 CAIX expression has been demonstrated in multiple tumour types histologically, although reports of CAIX expression in PCa are conflicting.42–44 In relation to potential clinical utility, one study has found baseline serum CAIX prior to chemotherapy in males with CRPC correlates with overall survival but additional independent studies to support this are limited.45 The clinical utility of CAIX in PCa therefore requires further investigation.

As well as stabilising HIF, severe hypoxia down regulates global translation through rapid induction of the unfolded protein response (UPR)14 and reduces cell growth by inhibiting signalling flux through the major target of rapamycin complex 1 (mTORC1).47 A reduction in endoplasmic reticulum (ER) protein maturation capacity under hypoxia induces both PKR-like ER kinase (PERK) and X-box binding protein 1 (XBP1) dependent arms of the UPR. In the context of tumour progression, PERK activation promotes resistance to apoptosis,52 while XBP1 is essential for tumour cell survival and growth under hypoxic conditions.53 Some studies have reported UPR down-regulation in mouse models,54 while more recently, PERK and

Figure 2. Cellular responses to hypoxia: Under oxic conditions PHD2 adds hydroxyl groups to HIF1α. This facilitates E3-dependent ubiquitination (not shown) and subsequent proteasomal degradation. Hypoxia inhibits PHD2-mediated hydroxylation, allowing HIF1α to dimerise with HIF1β, translocate to the nucleus and mediate transcription of target genes. Hypoxia also induces the unfolded protein response. Under hypoxia the ER has reduced capacity to mediate protein maturation. The ER chaperone BIP binds misfolded proteins in the ER and releases the luminal domain of PERK, facilitating PERK auto-phosphorylation and activation. Subsequent phosphorylation of eIF2α attenuates its role in global translation initiation and leads to activation of the ATF4 transcription factor. Collectively, these hypoxic responses can suppress transcription and translation of components of multiple DNA repair pathways, including HR, MMR and BER. BER, base excision repair; eIF2α, eukaryotic initiation factor 2α; ER, endoplasmic reticulum; HIF1α, hypoxia inducible factor 1α; HR, homologous recombination; MMR, mismatch repair; PERK, PKR-like ER kinase; PHD2, proline hydroxylase D2.
HIFα, an upstream splicing mediator of XBP1, have been associated with poor prognosis and PSA recurrence in patients. The role of hypoxia induced mTORC1 suppression in tumour development is more convoluted. There are suggestions that by acting as a barrier to cell growth, hypoxia selects for cells with deregulated mTORC1 signalling and greater capacity for deregulated cell growth. This theory would be in accordance with more general observations of hypoxia selecting for cancer cell aggressivity. However, the clinical relevance of hypoxia mTORC1 suppression in PCa remains poorly understood. Alternatively, other studies suggest chronically hypoxic cancer cells are able to take advantage of a non-canonical cap-dependent pathway, which is both HIF-2α dependent and distinct from mTORC1 suppression, thus maintaining de-novo protein synthesis and tumour growth under chronic hypoxia. There are currently no direct links to PCa and general therapeutic approaches are at an early stage, but progress in this field should be followed closely.

**TARGETING OF HYPOXIC CANCER CELLS**

Targeting the cellular response to hypoxia has so far proved challenging. VEGF inhibitors are the most successful having been FDA approved in several settings, although are not efficacious in PCa. Meanwhile aminoflavone, a ligand which stimulates aryl hydrocarbon receptor dimerisation with HIFIβ and thus inhibits HIF1 dimerisation, produced promising preclinical results but has shown limited clinical utility. Similarly, the synthetic oligonucleotide EZN-2968 suppressed HIFα expression and reduced tumour size in a PCa xenograft model, but its clinical development has since been suspended. First in class HIF-2α inhibitors are currently being developed with some promise, but their use is currently limited to renal cell carcinoma and acquired resistance is already proving problematic. In relation to hypoxic immune dampening, immunotherapy has been largely disappointing in unselected populations of advanced PCa. However, recent evidence suggests CDK12 bi-allelic loss in mCRPC patients confers increased neoantigen burden and T-cell infiltration. This subset of patients may therefore benefit from immune checkpoint inhibition. Various other approaches have also been investigated, including CAIX inhibition and targeting the mesenchymal epithelial transition (MET) receptor tyrosine kinase. However, most of these approaches are still in early clinical development and are not focused towards PCa.

As well as altering cellular biological responses, hypoxia also induces a biophysical effect and as such hypoxic cells are more resistant to radiotherapy and standard cytotoxic chemotherapy. This is because these methods rely on the generation of free radicals to damage DNA, while drug distribution in poorly vascularised tumours represents a more global issue for drug efficacy. Much effort has therefore also been placed on devising direct hypoxia targeting strategies. Inhaled carbon monoxide has demonstrated improved prostate tumour oxygenation in mice and patients, and could be a useful radiosensitiser. A randomised trial for radiotherapy in combination with carbon monoxide and nicotinamide in bladder cancer has demonstrated benefit and a Phase Ib/2 trial has confirmed the safety of this combination in PCa. Meanwhile, bioreductive prodrugs such as evofosfamide and tirapazamine have yielded little success in the clinical setting. A newer AQ4N analogue, OCT1002, has however demonstrated anti tumour efficacy in CRPC xenograft models and it will be interesting to follow its potential progress into the clinic. Given hypoxia targeting approaches have largely failed to establish themselves into clinical practice to date, an alternative, but not mutually exclusive strategy, could involve co-targeting the...
genetic instability observed in hypoxic cancer cells. Hypoxia directly induces genomic instability. One mechanism which could underpin this interaction is a global suppression of DNA repair under hypoxic conditions. Under normal conditions, cells induce a network of signalling events in response to genotoxic stress known as the DNA damage response (DDR). This encompasses activation of DNA damage sensors such as ataxia-telangiectasia RAD3 related/ataxia- telangiectasia RAD3 related (ATR/ATM), induction of cell cycle check points and the mobilisation of DNA repair mediators to ensure errors are not passed to daughter cells. Hypoxia has been shown to suppress both transcription and protein synthesis of base excision repair (BER) and mismatch repair (MMR) components, thus inhibiting the resolution of single strand breaks (SSBs). Further studies have demonstrated functional repression in the repair of double strand breaks (DSBs) in response to chronic hypoxia, triggering increased residual DSBs in G1 and dysfunctional homologous recombination (HR) repair in S and G2 phases of the cell cycle. Although hypoxia can also trigger replication stress through suppression of oxygen sensitive components involved in nucleotide biosynthesis, subsequent activation of ATR/ATM, even in the absence of DNA damage, maintains replication fork integrity where free deoxyribonucleotides (dTTPs) are limited. The sequelae of hypoxia-modified DNA repair and damage response pathways increases the chances of cells with damaged DNA to escape cell death and propagate a mutator phenotype.

**THE POTENTIAL ROLE OF DEFECTIVE DNA REPAIR IN HYPOXIA-ASSOCIATED GENOMIC INSTABILITY**

Given the combination of elevated levels of hypoxia and genetic aberrations in PCa portends a uniquely poor prognosis, efforts have been made to assess whether hypoxia directly induces genomic instability. Hypoxia has been shown to suppress a range of important HR mediators in vitro including BRCA1, BRCA2 and RAD51. Meanwhile, an inverse relationship between hypoxia and RAD51 has been demonstrated in PCa mouse xenografts, although in situ analysis to support this is still awaited. Potential mechanisms underpinning hypoxic suppression of HR include HIF1α competition for binding the lesion. Alternative evidence suggests suppression is mediated through reduced translation efficiency which supersedes the global reduction in translation observed under hypoxia. The functional impact of hypoxic HR suppression at the cellular level is a persistence of DNA damage, reduced replicative capacity and sensitivity to DNA interstrand cross-linking agents. Importantly, these cells are also sensitive to PARP inhibitors similar to cells that are genetically deficient in HR proteins.

**HYPOXIC SUPPRESSION OF HR**

The most lethal form of genotoxic stress a cell can encounter are DSBs and these must be repaired to maintain cell viability. Cells predominantly repair DSBs by executing one of two principal repair pathways, either non-homologous end joining (NHEJ) or homologous recombination (HR). Alternative end joining and single-strand annealing have also been shown to mediate a small number of DSB repair events, but will not be discussed here in detail. Preference for one mechanism of repair over the other is dependent on the stage of the cell cycle and HR/NHEJ mediator protein competition for binding the lesion. NHEJ involves direct ligation of the DSB ends in a fast but error prone manner. It is active throughout the cell cycle, does not require a template strand for repair and is therefore susceptible to DNA insertions, deletions, substitutions and translocations where two independent DSBs are joined.

Alternatively a double strand break occurring in S or G2 phases during DNA replication can be repaired using HR, whereby the intact sister chromatid provides a template to direct repair. In this scenario, the DSB is detected by the MRE11A-NSB1-RAD50 (MRN) complex, triggering further recruitment of ATM, C-terminal binding protein interacting protein (CtIP) and BRCA1 are both interacting partners of the MRN complex and collaboratively facilitate end resection in a 5’-3’ direction. Single stranded DNA (ssDNA) is rapidly bound by replication protein A (RPA), before being displaced by BRCA2 mediated loading of the RAD51 recombinase. Partner and localiser of BRCA2 (PALB2) is responsible for localising BRCA2 to the DSB and BRCA2 is able to bind RAD51 through eight conserved BRC repeats in exon 11 to facilitate RAD51 loading, which promotes sister chromatid invasion and a joint molecule with a homologous segment of dsDNA.

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**SYNTHETIC AND CONTEXTUAL LETALITY: TREATMENT OPPORTUNITIES**

An exciting development in the last decade for treating BRCA mutated tumours is inhibition of PARP, an enzyme which catalyses PAR addition to nuclear acceptors. PARP1 has a defined role in single strand break (SSB) repair, whereby PARylation of targets such as histones and PARP1 itself mediates attraction of DDR machinery. Inhibitors of this process such as olaparib mediate their function by a combination of PARP1 inhibition and PARP1 trapping on the DNA lesion, thus triggering the accumulation of un repaired SSBs which are processed to DSBs if encountered during replication. Where HR is dysfunctional, e.g. in the case of BRCA1/BRCA2 mutated tumours, DSB accumulation leads to mitotic catastrophe and cell death in a process termed synthetic lethality (Figure 3). This is of great relevance for PCa given patients in particular carrying a germline BRCA2 (gBRCA2) mutation exhibit a highly aggressive genomic profile, poorer prognosis than matched non carriers, and reduced response to first line taxane chemotherapy in the metastatic setting. Two key trials investigating olaparib monotherapy in mCRPC patients with HR/DDR mutations respectively have recently demonstrated both antitumour activity and improved patient outcome, thus providing compelling evidence for genomic stratification in this setting.

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Figure 3. Synthetic and contextual lethality: Endogenous and exogenous stress induce both SSBs/DSBs. Inhibitors of PARP prevent repair of SSBs, which are further processed to DSBs if encountered at a replication fork. Tumours with HR mutations such as BRCA2 (synthetic lethal), or cancer cells which have disseminated away from the nearest blood vessel and exhibit hypoxic suppression of HR proteins (contextual lethal) are unable to efficiently repair DSBs using HR. This leads to DSB accumulation, mitotic catastrophe and selective tumour cell killing. DSB, double stranded breaks; HR, homologous recombination; PARP, poly ADP ribose polymerase; SSB, single stranded breaks.

(Figure 3). This effect has been demonstrated in-vitro where PARP1 deficient murine embryonic fibroblasts (MEFs) had a proliferative disadvantage compared with wild-type under hypoxia. Another study investigated the effect of prolonged PARP inhibition on the microenvironment in a BRCA wild-type mouse model. They show neoadjuvant exposure to olaparib significantly reduces tumour hypoxic fraction, again suggesting preferential targeting of hypoxic cells with contextual suppression of HR. Follow-up radiation therapy demonstrated enhanced tumour radiosensitivity in olaparib pre-treated tumours, while ex-vivo cultures from these animals subsequently displayed decreased clonogenic survival in response to radiation, indicating hypoxic cells were being specifically targeted.

If replicated in PCa patient cohorts, these findings would provide an exciting therapeutic avenue to target hypoxia and treat patient subgroups with unstable genomes and uniquely poor prognoses. A key outstanding question here remains as to how hypoxia additionally impacts individuals who have existing HR alterations. For example, in the case of BRCA2 mutated PCa patients, hypoxia could suppress protein expressed from the remaining BRCA2 allele, thus inducing a contextual loss of heterozygosity (LOH) which underpins aggressive clinical course and a mutator phenotype. This would also shed light on why more than 50% of gBRCA2 localised PCa carriers retain the 2nd BRCA2 allele, yet still display clinical aggressivity. If proven, it would be predicted that patients with HR alteration and tumour hypoxia will be hypersensitive to PARP inhibition.

RATIONAL COMBINATIONS FOR ANTI-HYPOXIA THERAPY AND FUTURE OUTLOOK

Hypoxia is present in prostate and other solid tumours and has the capacity to drive tumour progression via both HIF dependent and independent signalling pathways. Multiple hypoxia biomarkers have demonstrated prognostic significance in PCa patients and its incorporation into patient treatment decisions should now be considered. As such, a 28-gene hypoxia signature specific for localised PCa patients has recently demonstrated prognostic benefit in retrospective cohorts and will be important for the stratification of these patients. This signature must now be confirmed in an appropriately controlled clinical trial before clinical deployment. Another interesting development was the recent finding from the STAMPEDE trial where radiation of the prostate improved response to ADT in newly diagnosed metastatic PCa patients.95 The biological mechanisms underpinning this require further understanding. As such, the HYPROGEN trial in Manchester (IRAS No. 262789) is exploring the potential of primary prostate tumour hypoxia driving early metastatic spread (Figure 4). The results from this study will reveal whether hypoxia and associated genomic instability are paired between the primary tumour and secondary metastatic sites, and...
thus provide further evidence for hypoxia driving an aggressive disease trajectory in PCa patients.

When considering direct targeting of hypoxia, there has so far been little success in PCa and focussing on hypoxia-induced vulnerabilities in DNA repair may represent a more feasible therapeutic avenue. It will be important to understand whether the combination of an existing HR alteration and hypoxic suppression of HR proteins has an additive effect on HR deficiency, potentially through a contextual LOH. This will require the development and concomitant use of biomarkers that measure hypoxia and DNA repair defects within patients’ prostate tumours as a selection tool for patients entering trials that are designed to co-target hypoxia and genetic instability. With this, we can also begin to explore additional concepts such as combining radiotherapy with anti-hypoxia agents or DNA repair inhibitors to improve therapeutic ratio in patients presenting with both genomic instability and tumour hypoxia (Figure 5).

Isogenic models, in particular for PTEN and BRCA2, are also required to functionally assess downstream events that occur in the presence or absence of tumour hypoxia. More generally, we must comprehend the primary order of events as to whether hypoxia drives genomic instability, e.g. through impaired DNA repair as covered here, or vice versa whether an existing unstable genome further drives tumour hypoxia through altered angiogenesis following cancer driver activation. Collectively, these studies will allow us to further improve hypoxia-associated prognostic signatures, more efficiently stratify localised PCa patients onto appropriate treatment regimens, and therefore better treat patients with aggressive hypoxic tumours.

**Figure 5. Assays and treatment for hypoxia mediated aggression:**

(a) Clinical outcome studies following surgery or radiotherapy for localised prostate cancer have shown that the patients whose prostate cancers acquire both high PGA and increased hypoxia have adverse outcomes when compared to patients who have only one or none of these two biological states.27,28 (b) Intraprostatic hypoxia subregions can be visualised in-situ using intrinsic (e.g. staining for HIF-1, GLUT-1) or extrinsic (e.g. pimonidazole binding) biomarkers, or metabolic imaging techniques such as OE-MRI and CSI-MRI.37 To date, imaging with PET hypoxic tracers (e.g. PET-FAZA or PET-MISO) has been less successful. Ascertainment of genetic instability or DNA repair deficiencies can be accomplished using genome sequencing techniques for CNAs or single nucleotide mutations. In-situ staining showing reduced DNA repair protein expression (e.g. reduced RADS1) relative to hypoxic staining may be a biomarker of hypoxia-mediated DNA repair deficiencies.27 Treatments to target hypoxia-mediated aggressive biology and improve cure in localised prostate cancer with surgery or radiotherapy includes the use of direct hypoxic cell toxins (e.g. evofosfamide and OCT1002) or radiosensitisation during radiotherapy using radiosensitisers (e.g. nimorazole, an oxygen mimetic) or increasing tumour oxygen content (e.g. carbogen and nicotinamide). DNA repair-deficient or cell cycle checkpoint-deficient hypoxic tumours can be targeted with PARP or ATR inhibition.98,99 Additionally, androgen deprivation has been shown to increase oxygen content and decrease DNA repair in prostate cancer.100,101 (c) These treatments can be used in neoadjuvant or concurrent settings to clear resistant and genetically unstable hypoxic cells within the primary tumour in combination with radiotherapy or surgery.94 Adjuvant treatments can improve outcomes by targeting occult metastatic disease and therefore prevent the outgrowth of lethal CRPC and NEPC metastases.22 Note concurrent use of DNA repair inhibitors with radiotherapy is generally too toxic to normal tissues and therefore neoadjuvant or adjuvant use of these agents may be preferred. CNA, copy number alteration; CRPC, castrate-resistant prostate cancer; CS-MRI, chemical shift MRI; NEPC, neuroendocrine prostate cancer; OE-MRI, oxygen-enhanced MRI; PET, positron emission tomography; PGA, percent genome alteration.
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