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Title: Integrating tumor hypoxic stress in novel and more adaptable strategies for cancer immunotherapy

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Abstract

Immunotherapy is poised to become an increasingly utilized therapy in the treatment of cancer. However, several abnormalities in the tumor microenvironment (TME) that can thwart the efficacy of immunotherapies have been established. Microenvironmental hypoxia is a determining factor in shaping aggressiveness, metastatic potential and treatment resistance of solid tumors. The characterization of this phenomenon could prove beneficial for determining a patient's treatment path and for the introduction of novel targetable factors that can enhance therapeutic outcome. Indeed, the ablation of hypoxia has the potential to sensitize tumors to immunotherapy by metabolically remodeling their microenvironment. In this review, we discuss the intrinsic contributions of hypoxia to cellular plasticity, heterogeneity, stemness and genetic instability in the context of immune escape. In addition, we will shed light on how managing hypoxia can ameliorate response to immunotherapy and how integrating hypoxia gene signatures could play a role in this pursuit.

Keywords: Hypoxia, immunotherapy, tumor, microenvironment, hypoxia signature

1. Introduction

In the last two decades, immunotherapy has emerged as an evolutionary approach for reviving the immune response against tumors in cancer patients. The clinical application of immune checkpoint blockers (ICB) against T cell receptors CTLA-4 (cytotoxic T-lymphocyte antigen 4), PD-1 (programmed death receptor 1) or the ligand PD-L1 (programmed death-ligand 1), has reshaped the treatment path of multiple tumor types [1-4]. Unfortunately, only a subset of patients successfully responds in the majority of tested cancers [5]. A substantial amount of data supports the crucial role played by the tumor microenvironment (TME) in determining the efficacy of ICB [6-10]. A prevalent characteristic of the microenvironment of solid tumors is the presence of low oxygen levels, defining a hypoxic state [11]. This phenomenon is a main factor for tumor progression and both chemo- and radiotherapy resistance in cancer [12, 13]. In addition, tumor hypoxia has been shown to contribute to resistance to immunotherapy in preclinical models, and hence, could offer novel clinical biomarkers for guiding treatment strategies [11, 14-16].

Three basic immune profiles can be distinguished in the TME of cancer patients, namely immune inflamed (“hot”), immune excluded and immune desert (“cold”) tumors [17]. While hot tumors include swarms of immune cells in the tumor parenchyma, in particular, CD4 and CD8 expressing T cells, immune-excluded tumors exhibit immune T cells in the stroma only, at the tumor margins, but not in the core. Meanwhile, cold immune desert tumors have a non-inflamed TME characterized by a few T cells. Clinical responses to ICB are more likely to occur in hot tumors. However, this response is not always observed, indicating that the presence of immune-cell infiltration is not the sole factor for defining clinical benefit from anti-cancer therapy [17].

Hypoxia is an inherent feature of the TME that arises from a skew in the balance between oxygen supply and consumption [18]. It is a key player governing various cancer hallmarks, including metabolic reprogramming, extracellular matrix (ECM) remodeling, epithelial to mesenchymal transition (EMT), angiogenesis, invasion, metastasis, cancer stem cell maintenance, immune evasion and genetic instability [19, 20]. At the molecular level, the hypoxia-induced malignant chain of events is primarily executed by activating the master regulator of oxygen homeostasis, HIF-1. HIF-1 is a member of the HIF (hypoxia-inducible factor) family of transcription factors, which also encompasses HIF-2, and HIF-3. The active HIF-1 functions as an α - β heterodimer composed of an oxygen-labile α -subunit (HIF-1 α) and a stable β -subunit (HIF-1 β) [21, 22]. Under normal oxygen levels, the α -subunit is hydroxylated by HIF-specific prolyl-hydroxylases (PHDs), driving its Von Hippel Lindau tumor suppressor protein (pVHL)-mediated proteasomal degradation [22]. Under hypoxic conditions, HIF-1 α is stabilized and interacts with HIF-1 β . The resulting HIF-1 binds the hypoxia responsive element (HRE) of target genes, thus mediating the

switch from oxygen-dependent to anaerobic metabolism [22]. With respect to its effect on tumor immunity, hypoxia has been reported to induce immune suppression and evasion through multiple mechanisms, including acidification of the TME, attraction of immunosuppressive cells and suppression of T-cell effector function [23, 24]. In addition, hypoxia promotes T-cell exclusion from the TME by stimulating the formation of leaky, malformed blood vessels [23-25]. Targeting hypoxia in conjunction with ICB has shown encouraging results in *in vivo* models of lung and breast cancer, melanoma, colon adenocarcinoma, as well as prostate cancer [14-16]. Moreover, hypoxia gene sets are enriched in melanoma tumors of non-responding patients to anti-PD-1 immunotherapy [26]

The characterization of tumor hypoxia is beneficial for determining a patient's treatment path and for the introduction of novel targetable factors that can enhance therapeutic outcome. The complex transcriptional response to hypoxia has been investigated in a myriad of studies. A fraction of these studies also reported a direct link between hypoxia gene signatures and patient prognosis, as well as response to hypoxia modifying therapy, in multiple tumor types [27-34]. Such studies have highlighted the clinical utility of hypoxia gene signatures by showing that the expression levels of genes within a signature could be combined to produce a hypoxic score, based on which the hypoxic state of a tumor is defined. By applying this direct and simplified approach, the patient's tumor itself could guide cancer treatment decision-making.

Herein, we discuss the intrinsic features of a hypoxic TME as it pertains to cellular plasticity, heterogeneity, stemness and genetic instability. In addition, we will be shedding light on how managing hypoxia can ameliorate response to ICB and the advantage of integrating hypoxia gene signatures in this endeavor.

2. Hypoxia in the Tumor Microenvironment

a. Hypoxia, tumor plasticity and heterogeneity

Tumor heterogeneity exists in most tumors with inherent effects on both their temporal evolution, and response to treatments [35-38]. It should be noted that both heterogeneity in the tumor, as well as, in the TME can greatly influence treatment responses. For instance, the immune content in the TME may dictate response/resistance to immunotherapies [39, 40]. On the other hand, cancer cells can undergo molecular and phenotypic changes referred to as cellular plasticity, which contribute to tumor heterogeneity, thus impacting both tumor progression and response/resistance to anticancer treatments, including immunotherapies [41-44].

A well-known example of such changes is reflected by the EMT program, which is a transdifferentiation program that governs changes of cellular states along the epithelial - mesenchymal spectrum [45-47]. The latter program is accompanied by epigenetic and transcriptional rewiring while conferring cells the capacity to switch between epithelial and mesenchymal states during tumor evolution, at least to a certain extent [41, 45, 48]. This EMT results in the acquisition of resistance to therapy [49-51].

A critical factor to consider is hypoxic stress, which is generated by insufficient concentration of oxygen, leading to phenotypic, metabolic, and epigenetics reprogramming of a cell, or a group of cells, thus acquiring a novel cellular state affecting their functions, as well as, interactions with neighboring cells [23, 52]. Hypoxia is to be considered a potent inducer of cellular plasticity in that it can promote the ability of a cell to shift from its original cellular state to a novel one. Evidence exists of a link between hypoxic stress and EMT in human carcinomas [53, 54]. Figure 1 represents a snapshot of the transformative impact of hypoxia in the TME on tumor plasticity, heterogeneity and immune resistance, in which the drop in oxygen levels (hypoxia) gives rise to a cascade of phenotypic changes resulting in increased tumor plasticity and heterogeneity. A subset of transformed cancer cells takes on mesenchymal features, reflected by a high EMT score resulting in their increased resistance to immune cell attacks and an increase in the recruitment of immune suppressive cells.

As it is now demonstrated by numerous experimental paradigms, both tumor plasticity and heterogeneity are major determinants in the emergence of therapy resistance [37, 55-57]. In this regard, numerous mechanisms associated with EMT of carcinoma cells are proposed to explain tumor immune escape [57-59]. First, EMT can render cancer clones poorly immunogenic. In human and mouse cancer systems, the acquisition of a more mesenchymal phenotype by cancer cells has been associated with deficiencies in MHC class I antigen presentation [60-62]. In human lung cancer tissues, reduced amounts of immunoproteasome components were reported [63]. Second, in various models, a more mesenchymal phenotype has been linked to reduced recognition by immune cells following loss of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) [64, 65]; or associated with defects in the establishment of immunological synapses. This could be explained in part by altered actin network [66], or tyrosine phosphorylation signaling in the vicinity of the cell-cell contacts [54].

On the other hand, one should consider the possibility that in certain cellular contexts EMT induction could increase cancer cell susceptibility to natural killer (NK) cells, especially when EMT is associated with up-regulation of natural killer group 2D (NKG2D) ligands or cell adhesion molecule 1 (CADM1), as well as modulation of MHC class I and E-cadherin amounts [67, 68]. Additionally, mechanistic studies showed that the resistance of tumor cells with overexpression of brachyury (a transcription factor

associated with tumor EMT) is due to inefficient caspase-dependent apoptosis [69]. EMT-associated autophagy was also described as a tumor resistance mechanism [50] in conjunction with various molecular perturbations identified in resistance to T-cell-mediated killing of tumor cells [69-72].

Moreover, tumor resistance mechanisms directly involving HIF-1 α have been reported. Zhang and colleagues [73] found that HIF-1 α stimulates CD47 expression. CD47 is a significant player in cellular plasticity, that additionally aids in the avoidance of phagocytosis in breast cancer cells [74]. This is achieved by hampering the “eat me signal” on cancer cells through the interaction of CD47 with the signal regulatory protein (SIRP) on macrophages, thereby impairing phagocytosis. Noman and colleagues [75] have demonstrated that EMT-dependent up-regulation of CD47 inhibited phagocytosis of EMT-activated mesenchymal cancer cells. Taken together, these data provide insight into how cancer cell plasticity and HIF-1 α may drive multi-resistant phenotypes including resistance to radiotherapy, chemotherapy and immune resistance.

Furthermore, in hepatocellular carcinoma, hypoxia-induced EMT triggered an immunosuppressive TME in association with impairment of T-cell proliferation and promotion of regulatory T-cell (Treg) expansion, through the induction of indoleamine 2, 3-dioxygenase (IDO) expression in monocyte-derived macrophages [76]. Indeed, many studies highlighted the central role of IDO as an inhibitor of the antitumor immune responses [77, 78]. Nevertheless, despite initial promising results in early-phase clinical trials in a range of tumor types, a phase III study of the IDO1-selective inhibitor combined with pembrolizumab showed no difference between the IDO inhibitor-treated group versus placebo in metastatic melanoma patients [79]. In another study, secreted protein, acidic and rich in cysteine (SPARC) expression, a well-known mediator and marker of EMT, contributed to the establishment of an immunosuppressive microenvironment, composed of infiltrating Tregs, mast cells, and myeloid derived suppressor cells (MDSCs) [80].

Finally, it was shown that a high EMT signature score correlated with increased tumor infiltration of immunosuppressive CD4⁺ Foxp3⁺ Tregs, and an upregulated PD1/PDL1 axis components [81]. More importantly, a survey inspecting tumors of different origins revealed positive correlations between an EMT signature score, and the amount of inhibitory immune checkpoints PD-1, PD-L1, PD-L2, OX40L and CTLA4 [82].

Taken together, these data provide insight into how plasticity of cancer cells may drive immune resistance phenotype. More studies are now needed to decipher how EMT mediators control immunomodulatory genes and immune environment during tumor progression, as well as response to immunotherapies.

b. Hypoxia interferes with cancer cell stemness

Both hypoxia and HIF signaling pathway are essential in the regulation and sustenance of cancer stem cells (CSCs), and EMT phenotype [83, 84]. Thus, hypoxia in the TME promotes the neoplastic process by, at least, generating a CSC-like phenotype. CSCs, referred to as “tumor initiating cells” or “tumor propagating cells”, are cancer cells with properties of normal stem cells. They share characteristics with mesenchymal cells and may be responsible for tumor initiation, invasive growth, and metastasis development [85]. As they possess self-renewing properties that drive long-term tumor survival, CSCs have been hypothesized to be the root cause of malignant cancers, and to contribute to cancer aggressiveness. Hypoxia results in an increase in transcription factors that support the stem-like state of cells, namely, OCT4, SOX2, c-myc, and Nanog [84, 86, 87]. Nanog is a prominent and pluripotent factor in the activation of autophagy under hypoxia, participating in the increase of tumorigenic potential in the setting of hypoxia-induced tumor cell resistance. While Nanog is involved in the maintenance of self-renewal and pluripotency of embryonic stem cells, it also plays a role in immunosuppression by directly regulating TGF- β 1 that modulates the expansion of Tregs and that increases macrophage recruitment, enabling tumor evasion of the immune system [88]. Nanog also contributes to the induction of autophagy in hypoxic tumor cells, and to their resistance to killer cells in the TME [89].

Additionally, unique surface markers are overexpressed on CSCs that are significantly correlated with tumor growth and proliferation. Currently, several promising therapeutic strategies targeting CSCs exist, including blocking both oncogenic signal transduction pathways and telomerase activity in the CSCs; inducing the CSCs differentiation and microenvironmental modification; as well as using specific gene therapy [90, 91]. Furthermore, several studies revealed that the sensitivity of cancer cells to chemotherapy can be enhanced by inhibiting key CSC markers such as Nanog, ATP-binding cassette transporters (ABC transporters), Wnt, CD44, CD133, CD55, ALDH1, OCT4, SOX2 or KLF4 [92-94]. Hence, the CSCs markers are crucial for identifying and developing novel targeted therapies. These therapies could potentially reduce tumor recurrence, metastasis, and drug resistance. However, the development of such therapies may be entangled by off-target effects, which result in normal tissue damage and treatment-induced toxicity [95]. CSCs have heterogeneous phenotypes as a result of their adaptation and cross-talks with the hypoxic TME following therapeutic pressures [90]. They express surface stemness-related markers potentially exploitable in cancer treatment, including immunotherapy [96]. However, CSCs

express immunosuppressive molecules (i.e. CTLA4, B7-H2, B7-H3, PD-1/-1L) [97, 98], low levels of MHC-1 [99] and can produce higher level of macrophage migration inhibitory factor (MIF) [100]. Thus, they are characterized by low immunogenicity and immunosuppressive activity [101].

Another cellular pathway contributing to the CSCs immune resistance is the JAK/STAT pathway that is deregulated in these cells. In fact, STAT3 signaling is an important regulator of CSCs in tumor formation [102], invasion and progression [103]. While, STAT3 activation is tightly controlled, activated STAT3 (p-STAT3) has been identified in many kinds of human tumor samples and cancer cell lines, underlining its pivotal role in tumor development and malignancy [103-106]. As such, inhibiting STAT3 triggers an intrinsic immune-surveillance response that curbs the growth of cancer cells and augments antitumor immunity [107, 108]. Consequently, several ongoing trials are targeting the STAT3 signaling pathway including Napabucasin (BBI608), which affects cancer cell stemness by inhibiting oncogenic cellular pathways, which is currently in phase III clinical trial and is under investigation in metastatic colorectal cancer, metastatic pancreatic cancer, and other solid tumors [109, 110]. In mouse models, napabucasin was effective both as a monotherapy and in combination with other agents; in particular, synergy was observed with paclitaxel *in vivo* [111].

We should note, however, that targeting STAT3, presents challenging roadblocks in preclinical models and early-phase clinical trials for solid malignancies mainly due to the lack of full inhibition of STAT3, off-target effects, the lack of specificity and/or potency, and the lack of comprehensive understanding of the intricate signaling crosstalk [112, 113]. As such, to improve its pharmacokinetics properties, synthesis of napabucasin derivatives that display stringer inhibitory activities are being designed [114].

In addition to the STAT3 targeting drug listed above, drugs that target cell surface markers (including CD44, CD47), cell signaling pathways (including Wnt, Notch, Hedgehog, PI3K/Akt) and that modulate the immune system (CXCR4 antagonists in combination with PD-L1 drugs) have entered clinical trials [110]. Of these, Enoticumab, an antibody to DLL4, a Notch ligand expressed specifically in endothelial cells and that plays a vital role in regulating angiogenesis, is having promising clinical activity in phase I clinical trials for the treatment of advanced solid malignancies [115]. Another small molecule inhibitor that targets the hedgehog signaling pathway, vismodegib, has gained FDA approval for the treatment of advanced basal cell carcinoma while its use for other types of cancers is at various stages and has shown promising activity in triple negative breast cancer CSCs [116]. Effective targeting of CSCs may require a combination type of therapy, and personalized medicine approach will ultimately determine the most appropriate anti-CSC agent used. Indeed, anticancer therapy resistance is only partially reversed following targeted therapy toward these above-mentioned CSCs mechanisms. The partial efficacy to these CSC-targeted strategies points towards the existence of additional underlying determinants for CSCs

resilience [117]. Finally, to be most effective, anticancer drugs must efficiently penetrate the tissue to reach all the cancer cells at adequate concentrations to exert their effect. The distance between solid tumors hypoxic regions and blood vessels results in a limited distribution of the drugs, which limits their effectiveness.

c. Hypoxia-induced DNA damage response

Most solid tumors experience hypoxia that are of acute, chronic and/or fluctuating type, characterized by low pH and nutritional imbalance. To manage such extreme stress and maintain cellular homeostasis, hypoxic cells conserve ATP by decreasing the proliferation rate and DNA repair capabilities. Hypoxic cells exhibit reduced DNA capacity through transcriptional and translational downregulation of DNA replication and repair proteins. Although common DNA damage and repair assays like comet assay, pulsed-field gel electrophoresis, absence of phosphorylated H2AX and 53BP1 foci demonstrate the lack of DNA damage under hypoxic conditions, the DNA damage response is activated remarkably. This is evidenced by the phosphorylation Ataxia Telangiectasia Mutated (ATM) and ATM-Rad3-related (ATR) and pan nuclear distribution of γ H2AX inside the nucleus [118]. Depending upon the duration and severity of hypoxia, DNA repair pathways can be affected at different rates. Acute hypoxia leads to activation of DNA damage response through post-translation modifications. More chronic hypoxia leads to downregulation of mismatch repair capacity, double strand break repair (homologous recombination and Non-homologous end-joining) and nucleotide excision repair [118]. Further, longer duration of hypoxia poses serious threat to DNA repair mechanisms through epigenetic modifications in critical DNA repair genes like MLH1 and BRCA1. In addition, severe hypoxia can lead to replication stress, characterized by stalled replication forks and accumulation of long single-stranded DNA threads. Replication stress further triggers the ATM and ATR signaling to activate the DNA damage response [119].

Strategies for targeting DNA repair mechanisms in hypoxic cells are multifold. It is possible to target the post translational modifications in acute hypoxic phase through kinase inhibitors. Radiotherapy and/or PARP inhibitors can intensify DNA damage in chronic hypoxic cells that are already compromised for DNA repair capabilities [120]. Further, integrating DNA damaging agents with immune checkpoint blockade provides a rationale for targeting hypoxic cells with underlying DNA repair deficiency [121].

d. Hypoxia induced genetic instability and immunogenicity

Hypoxia-induced genetic instability can be attributed mainly to aberrant DNA damage signaling and compromised DNA repair capacity. Hypoxic tumor cells are affected at the DNA level by excessive

hypoxia and reoxygenation -induced reactive oxygen species (ROS) and decreased mitochondrial ATP production, inducing DNA damage checkpoints alteration during the cell cycle [122]. In addition, hypoxia-mediated genetic instability is evidenced by increased rate in point mutations, deletions, insertions, translocations and duplications as well as modifications in fragile sites [123]. An increase in microsatellite instability has been shown in cells with hypoxia-induced downregulation of the MMR pathway [124]. The double strand break (DSB) repair pathways by hypoxia can have severe consequences on genome integrity. For example, the induction of double minutes and expression of fragile sites has been seen in hypoxic cells leading to gene amplifications [125]. Hypoxia can exert selection pressure on tumor cells with a mutator phenotype through reduced apoptotic capacity [126]. Even at low hypoxic levels (24h at 0.2% O₂), tumor cells with alterations in DNA sequence can bypass the G1/S and G2/M DNA checkpoint kinases, ATM and ATR [127], thus contributing to genomic instability.

Hypoxia-induced genetic instability may have some important consequences on tumor immunogenicity as well as response to immunotherapy. Both oxidative DNA damage and DSB regulate the expression of PD-L1 in cancer cells [128, 129]. Moreover, intrinsic DNA damage response can induce interferon response, activating the immune system [130, 131]. Nevertheless, the contribution of hypoxia or reoxygenation-induced DNA damage on the immune response is yet to be understood. Another important (but not fully described) aspect of the DNA damage response is to enable antigen-presenting functions [132]. In murine and other tumor models, contradictory reports about the up- and downregulation of hypoxia-induced MHC class I molecules on tumor cell surface were outlined [133, 134]. A diverse repertoire of tumor-specific neoantigens arises as a consequence of tumor-specific mutations (via non-synonymous somatic mutations that modify amino acid coding sequences) [135]. These neoantigens represent a major factor in the recognition and rejection of cancer cells by the immune system. As such, neoantigens emerge as a solid parameter in the clinical activity of cancer immunotherapy. Tumors expressing neoantigens seemingly exemplify suitable targets for T cell-based cancer immunotherapy [136]. In fact, tumors expressing neoantigens have increased immunogenicity as there is increased homing of neoantigen-specific T-cell pool at the tumor site and neoantigen-specific T cells are not subject to central and peripheral tolerance [137]. Positive correlation of tumor mutational burden/neoantigen load and immunotherapy response has been well-established [138]. In addition, it was shown that hypoxia was associated with increased genomic instability exhibiting inter- and intratumor heterogeneity [139]. Finally, tumors with mutations in DNA repair genes and/or inactivated DNA repair pathways can boost neoantigen load [140, 141].

As hypoxia can influence DNA repair pathways and contribute to genomic instability in the tumor microenvironment, it will be important to understand the effect of hypoxia alone on the mutational spectrum and neoantigen load, and the latter's influence on immunotherapy.

3. Clinical impact of hypoxia on immunotherapy

The abnormal TME, which is characterized by hypoxic, mal-vascularized, acidic and nutrient-deprived conditions has proven to be a prominent barrier to ICB, as it attracts immunosuppressor cells and excludes cytotoxic T cells or inactivate their effector function [24, 142]. Indeed, strategies for normalizing the TME have been shown to enhance the efficacy of ICB in mice with primary and metastatic breast cancer [9, 143, 144] and have been thoroughly reviewed [11, 25]. Hypoxia itself plays a pivotal role in sculpting the tumor reactive stroma by regulating tumor resistance, heterogeneity and plasticity, and immune suppression. As such, the characterization of tumor hypoxia is beneficial for determining novel targetable factors that can enhance patients' therapeutic outcome.

The relevance of hypoxia in resistance to immunotherapy has further been corroborated by a study of gene signatures that correlated resistance to PD-1 antibody therapy in melanoma patients with the enrichment of hypoxia gene sets in non-responding patients [26]. Similarly, a study by Buart S. and colleagues [145] revealed that the expression levels of hypoxia-regulated BNIP3 (Bcl-2 adenovirus E1B 19 kDa-interacting protein 3)/GBE1(glycogen branching enzyme1) differential pair to be significantly associated with response status of a small cohort of melanoma patients to anti-PD1 (pembrolizumab) immunotherapy. *BNIP3* and *GBE1* are two genes identified from a 35-gene hypoxic signature validated in primary melanoma cell lines and patient-derived tumor tissue [145].

Recently, targeting hypoxia-mediated factors has been reported to sensitize tumors to immunotherapy *in vivo* [14-16, 146]. The expression of Carbonic Anhydrase IX (CAIX or CA9) hypoxia-induced enzyme, which is a cell-surface pH regulatory enzyme, has been correlated with a reduced Th1 response in a broad spectrum of tumors and with a worse overall survival in some cancer patients [146]. In particular, targeting CAIX with a specific small molecule in cancer patients, reduced both the tumor cells' glycolytic metabolism and TME acidification, resulting in increased immune cell killing and enhanced locally and systemically ICB activity [146]. Indeed, under hypoxic conditions, tumor cells increase the level of HIF-1 α and HIF-2 α , thus upregulating the expression of CAIX and activating glycolysis [147]. These various data suggest that pharmacologically inhibiting CAIX, in combination with ICB, is a potential therapeutic strategy for enhancing immune activity, via a better Th1 response. Hence, in patients with hypoxic solid tumors, blocking CAIX, in addition to ICB, may decrease tumor growth, and reduce metastasis, therefore improving the overall survival of cancer patients.

Apart from pH neutralization, hypoxia reduction by directly altering tumor oxygenation has been investigated in conjunction with ICB [14, 15]. Hatfield SM and colleagues [14] reported that respiratory hyperoxia improved pulmonary tumor regression induced by dual blockade of CTLA-4 and PD-1. Hyperoxia in tumor-bearing mice subjected to 60% oxygen led to enhanced intratumoral infiltration and reduced inhibition of cytotoxic T lymphocytes (CTLs). On the other hand, the inhibition of both oxygen consumption and subsequent tumor-induced hypoxia, using metformin, has been associated with a better efficacy of anti-PD1 immunotherapy [15]. These findings suggest that the normalization of tumor-induced hypoxia with metformin generates a more permissive TME to anti-PD1 immunotherapy, thus allowing an improved antitumor immune response, promoting tumor regression [15].

Hypoxia-activated prodrugs (HAPs) have been developed to specifically target hypoxic tumor cells. The integration of HAPs to sensitize “cold” tumors to ICB has proven to be a promising approach in mouse models of metastatic prostate cancer [16]. Despite the encouraging results of several HAPs-based preclinical and clinical phase I and II trials, their implementation in the clinic has, thus far, been unsuccessful [148]. This could be due to the challenge in delivering these compounds to their target cells, which lie in hypoxic zones that are distant from functional blood vessels [149]. In addition, another central limitation is patient stratification [149]. Indeed, a better way to tackle the HAPs-based therapies could be a Phase III study design that is biomarker-stratified; such that only patients who are biomarker-positive, that is hypoxia-positive, are randomized between ICB treatment and an ICB-HAPs combination treatment [148].

Therefore, there is currently a critical need for integrating the assessment of tumor hypoxia biomarkers in the clinic to improve patients’ response to ICB treatment [150, 151]. Nonetheless, and despite all the evidence linking tumor hypoxia with immunotherapy resistance, there are a few ICB-based trials assessing the effect of hypoxia on the efficacy of immunotherapy in cancer [152]. The apparent reluctance to build hypoxia-biomarker based anticancer trials is probably due to the difficulties in measuring tumor-induced hypoxia [152]. In this case, hypoxia gene signatures could potentially serve as a straightforward hypoxia-biomarker based therapeutic approach for patients undergoing immunotherapy.

4. Assessment of tumor hypoxia: the road to hypoxia gene signatures

One of the earliest methods to assess hypoxia was the direct measure of oxygen partial pressure (pO_2) in a patient’s tumor tissue in comparison with normal tissue, using fine needle electrodes [153, 154]. While this validated technique is considered the gold standard, it is challenged by several disadvantages. The latter include: the highly invasive nature of this technique, its inability to distinguish between hypoxic and necrotic cells, which do not contribute to tumor growth, and its limited application (this technique being restricted to accessible tumors) [155]. A non-invasive promising imaging approach to monitor tissue

oxygen levels has recently been developed, namely blood oxygenation level-dependent magnetic resonance imaging detection (BOLD-MRI) [156]. BOLD-MRI assesses tissue oxygenation by utilizing hemoglobin as an endogenous contrast agent and measuring deoxygenated hemoglobin content by sampling the tissue oxygen content. However, the signal is sensitive to other factors than hypoxia, thus limiting its specificity. To circumvent this issue, an indirect method involving the use of hypoxia exogenous markers, such as pimonidazole, was developed [155, 157]. An alternative hypoxia-imaging approach, which does not need an additional biopsy as in the pimonidazole-based method, is the use of hypoxia-specific positron emission tomography (PET) radiotracers [155]. Currently, the most extensively used PET tracer is ^{18}F -FMISO [158]. Another feasible method has been the immunohistochemical detection of hypoxia-induced endogenous protein markers, such as HIF-1, CAIX and VEGFA, which can be characterized on tumor biopsies [155, 159]. Nowadays, hypoxia gene signatures have emerged as novel means to capture the effect of hypoxia in cancer and translate it to prognostic and predictive biomarkers. These signatures offer a direct and simplified approach for determining a tumor's hypoxic state; in what follows, the most pertinent signatures will be discussed, as will their relevance to immunotherapy.

a. Prognostic hypoxia gene signatures

Over thirty-two hypoxia gene signatures, ranging from metagenes to compact gene lists, have been published to date [159, 160]. A fraction of these signatures additionally harbored clinically relevant prognostic potential (Table 1). Indeed, the eighteen presented signatures revealed a common consensus, where patients classified as having more hypoxic tumors experienced worse prognosis than those with less hypoxic tumors. Moreover, fourteen signatures were found to be independent prognostic factors (based on multivariate analysis), and at least five signatures demonstrated prognostic significance in multiple tumor types. Such signatures seemed to capture a transcriptional response to hypoxia that was reflective of prognosis in multiple cancers.

The methods utilized for deriving hypoxia gene signatures varied and could be summarized as distinct combinations of *in vitro*-, *in vivo*- and computational- based approaches [159, 160]. The hypoxic score of a tumor could be determined either from a continuous score, which is binarized into high- and low-hypoxia using a predetermined cut-off for the cohort median/mean score; or the hypoxic score could be used to directly binarize tumors into high- and low-hypoxia, as in the case of formula-based signatures. The resulting gene signature classification of the hypoxic state of a tumor is thus similar in its indirect nature to measurements done using immunohistochemical analysis of endogenous and exogenous markers of hypoxia; on the other hand, oxygen electrodes allow the direct measure of oxygen concentration. Overgaard's group, who derived a 15-gene hypoxia signature with prognostic power in head and neck

cancer, was the only one to pair the derivation of the gene signature with direct pO₂ measurements in patients using needle electrodes, thus directly associating the genes in the signature with the hypoxic phenotype *in vivo* [30]. This signature, which, was also found to be prognostic in soft tissue sarcoma (Table 1), poorly correlated with oxygen electrode measurements in 16 patients. As per the authors, this could be due to the small number of patients for which tumor oxygen tension status was available as well as the effect of necrosis and tumor size on pO₂ measurements [161]. The technically demanding and highly invasive nature of needle electrode pO₂ measurement makes it impractical to benchmark hypoxia gene signatures against this gold standard criterion in a large-scale validation study [159]. In a fraction of studies, other classical methods for the assessment of hypoxic status, such as imaging and immunohistochemical staining of endogenous or exogenous markers of hypoxia, were integrated in the production of gene signatures [162-165]. These studies highlighted the utility of hypoxia gene signatures in substituting traditional hypoxia biomarkers as a more practical approach for widespread clinical use [159].

b. Predictive hypoxia gene signatures

Among the published hypoxia gene signatures, four were found to be predictive of response to hypoxia modifying therapy [30, 32-34]. These signatures were retrospectively tested in phase III randomized trials of radiotherapy alone or in combination with: carbogen and nicotinamide (CON) as in the ARCON (accelerated radiotherapy with carbogen and nicotinamide) and BCON (bladder carbogen nicotinamide) trials, or with nimorazole, as in the DAHANCA 5 (The Danish Head and Neck Cancer) trial. Carbogen and nicotinamide are employed to increase oxygen supply and enhance response to radiotherapy. Meanwhile, nimorazole is a hypoxia-targeted agent that acts as a radiosensitizer. The predictive power of the signatures tested in these trials followed the same direction, wherein only tumors classified as hypoxic based on the adapted signature benefited from the inclusion of hypoxia modifying therapy with radiotherapy regimens.

In particular, the 26 top ranked genes from the Buffa signature [31] were able to predict benefit from CON in 157 patients with T2-T4 laryngeal cancer enrolled on the ARCON trial [32]. Hypoxia scores (HS) for each tumor were derived and patients were stratified into low HS and high HS groups based on the cohort median scores for the expression of all 26 genes. Laryngeal high HS patients showed greater benefit from ARCON, where the five-year regional control was found to increase from 81% with radiotherapy alone to 100% with CON (log rank test P=0.009). This trend was also achieved when a 24-gene bladder specific hypoxia signature was used to stratify 185 patients with locally advanced bladder carcinoma, enrolled on the BCON trial, into low HS and high HS groups [33]. Patients with high HS receiving CON with radiotherapy had an improved local progression free survival compared to those

undergoing radiotherapy alone (univariate Cox PH analysis $P=0.015$, HR 0.47, 95% CI 0.26–0.86) [33]. Interestingly, the 28-gene signature derived from prostate cancer cell lines was also predictive of hypoxia modifying therapy in the BCON trial, in which patients with tumors stratified as having high hypoxia had improved survival with the addition of CON (Cox PH analysis $P=0.021$, HR 0.54, 95% CI 0.32–0.91) [34]. On the other hand, the integration of CON in patients with low 28-gene signature scores was associated with worse survival (Cox PH analysis $P=0.037$, HR 2.49, 95% CI 1.06–5.89), and 25% of these patients suffered from CON's negative side effects [34]. This outcome further underlines the utility of signatures in guiding treatment strategies to avoid such unnecessary adverse effects.

The fourth predictive signature came from Overgaard's group, whose 15-gene hypoxia signature accurately predicted benefit from integrating nimorazole in the more hypoxic tumor fraction of 323 patients with HNSCC in the DAHANCA 5 trial. Indeed, higher cumulative incidence of locoregional failure at 5 years was observed in patients with more hypoxic tumors compared to those with less hypoxic ones (79% vs. 54%, $P=0.001$) [30]. This group has also validated the signature for prospective use in clinical trials [203]. Indeed, while all these signatures sound promising at the translational level, they have been derived and tested in a retrospective manner and their validation in prospective studies is pending. Nonetheless, the path seems promising, with two prospective trials already in progress for the 26-gene [32, 159] and 15-gene [30, 203] head and neck cancer signatures, respectively.

c. Merging signatures: Hypoxia gene signatures in the context of anti-tumor immunity

Cellular processes implicated in tumor progression and molecular classification, as well as clinicopathological factors, can be combined with hypoxia gene signatures to increase their prognostic and potentially predictive power in the clinic [139, 151, 204-208]. The same concept can be applied in the integration of factors representative of a tumor's immune microenvironment, thus adding another layer of scrutiny for tumor classification and patient stratification.

In this respect, it has been shown that in soft tissue sarcoma (STS), tumors with high HS have significantly lower immune infiltration scores than the low HS counterparts, suggesting that, hypoxia is associated with a weakened immune response [180]. Moreover, the most recently published studies have capitalized on the two interdependent characteristics of solid tumors, hypoxia and immune cells infiltration, to put forth signatures predictive of patient prognosis [151, 209].

Based on these studies it would be of great value to further derive and test the significance of combined hypoxia and immune signatures in predicting response to immunotherapy in a prospective fashion. In addition, it would be insightful to determine the pan-cancer vs cancer specific nature of such combined signatures and how they could be influenced by innate tumor heterogeneity.

5. Hypoxia targeting and hypoxia-alleviating drugs in immune intervention

Formulating effective immunotherapy to overcome a tumor's immune escape from immunosurveillance is a major challenge. Understanding hypoxia and its components that generate an immunosuppressive environment is crucial in developing more efficient immunotherapies.

Hypoxia associated abnormal vasculature facilitates immune evasion and reduces the efficacy of immunotherapy by decreasing delivery of oxygen, CTLs and drugs. Different, hypoxia-targeted and hypoxia alleviating therapies have been identified [210, 211]. These drugs can be used in combination with immune checkpoint blockers (CTLA-4, PD-1 and PD-L1) that have been approved by the FDA (Food and Drug Administration) for the treatment of diverse cancer types [3, 4, 212].

Drugs designed in an attempt to target HIF-1 α transcription factor are many, encompassing those inhibiting its DNA binding, protein synthesis, protein degradation and mRNA expression [213, 214]. Combinations of these drugs with immunotherapy may overcome hypoxic resistance, especially given that many classical chemotherapeutic agents, such as epirubicin, cisplatin, doxorubicin, and cyclophosphamide, are known immunogenic cell death inducers [215, 216]. Indeed, there are several such clinical trials being undertaken at the moment, and these are presented in Table 2. This table also highlights the mode of action of the hypoxia targeting drugs and the mechanisms being targeted.

On the other hand, normalizing vessel function may enhance tumor perfusion, thus supporting more homogeneous delivery of drugs, oxygen, and immune cells [217]. Several clinical and preclinical hypoxia-alleviating strategies have been shown to be beneficial through the combination of anti-VEGF/VEGFR2 agents with various immunotherapies [211]. For example, there are about 83 immunotherapeutic combinational clinical trials on Bevacizumab, a recombinant, humanized mAb to VEGF, including, for instance, a phase III trial in treating colorectal cancer (NCT02997228) and a phase II Non-small Cell Lung Cancer with Brain Metastases (NCT02971501). Three other phase III trials with PD1/PD-L1 antibodies and anti-VEGF or anti-VEGFR agents, have led to FDA approvals for the combination in lung and kidney cancers (NCT02684006, NCT02853331, and NCT02366143) [211]. Several other combination strategies that improve the function of tumor vessels have been reported and they are thoroughly reviewed elsewhere [11, 25, 142, 211, 218]. The hypoxia alleviating drugs that are currently being tested in combination with immunotherapeutic interventions in clinical trials are also listed in Table 2.

Overall, it should not be disregarded that vast challenges lie ahead, due to the complexity and heterogeneity of tumor hypoxia. Indeed, not all patients fully respond to hypoxia-targeted therapy, and several trials have been terminated due to the lack of efficiency and low response rate [219]. Combination

of hypoxia targeting drugs with immunotherapy using delivery nanoparticles could possibly overcome such drawbacks of targeting tumor hypoxia [220]. In addition, other challenges linked to resistance mechanisms and presence of suitable biomarkers for patient selection and monitoring in the clinic remain to be resolved [11].

6. Concluding remarks

Hypoxia in the tumor microenvironment plays a central role in the evolution of immune escape mechanisms by tumor cells through its impact on tumor heterogeneity and stroma reactivity. Strong evidence has been provided indicating that tissue hypoxia contributes to therapeutic resistance, heterogeneity and progression. It also interferes with immune plasticity, promotes the differentiation and expansion of immune-suppressive stromal cells, and modifies the metabolic landscape to support immune privilege. Manipulating host-tumor interactions in the context of the hypoxic TME may therefore be critical in reverting malignant conversion and in, presumably, guiding cancer treatment.

Gene signatures have emerged as a novel approach for defining the hypoxic state of a tumor and among existing hypoxia detection methods, they are in the lead for advancing to clinical application [159]. Such signatures could prove essential for patient stratification and guiding immunotherapy treatment approaches, in which hypoxia is a known culprit. Given that intratumoral hypoxia is a driving force of tumor progression, playing a critical role in remodeling the tumor stroma and favoring the emergence of immune tolerance, efforts to incorporate components of the hypoxic microenvironment are attracting particular attention at present in guiding the successful design of future cancer immunotherapeutic approaches.

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8. Conflict of interest statement

The authors declare no conflicts of interest.

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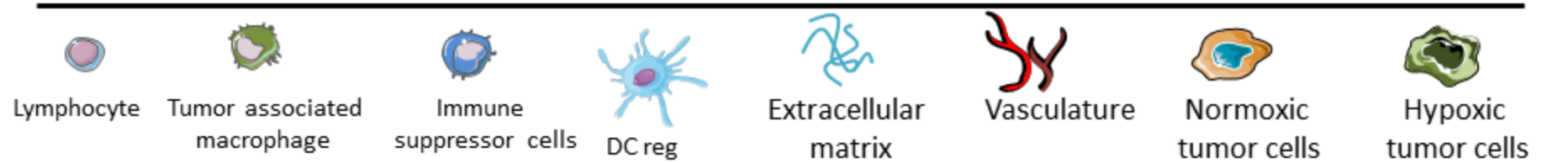
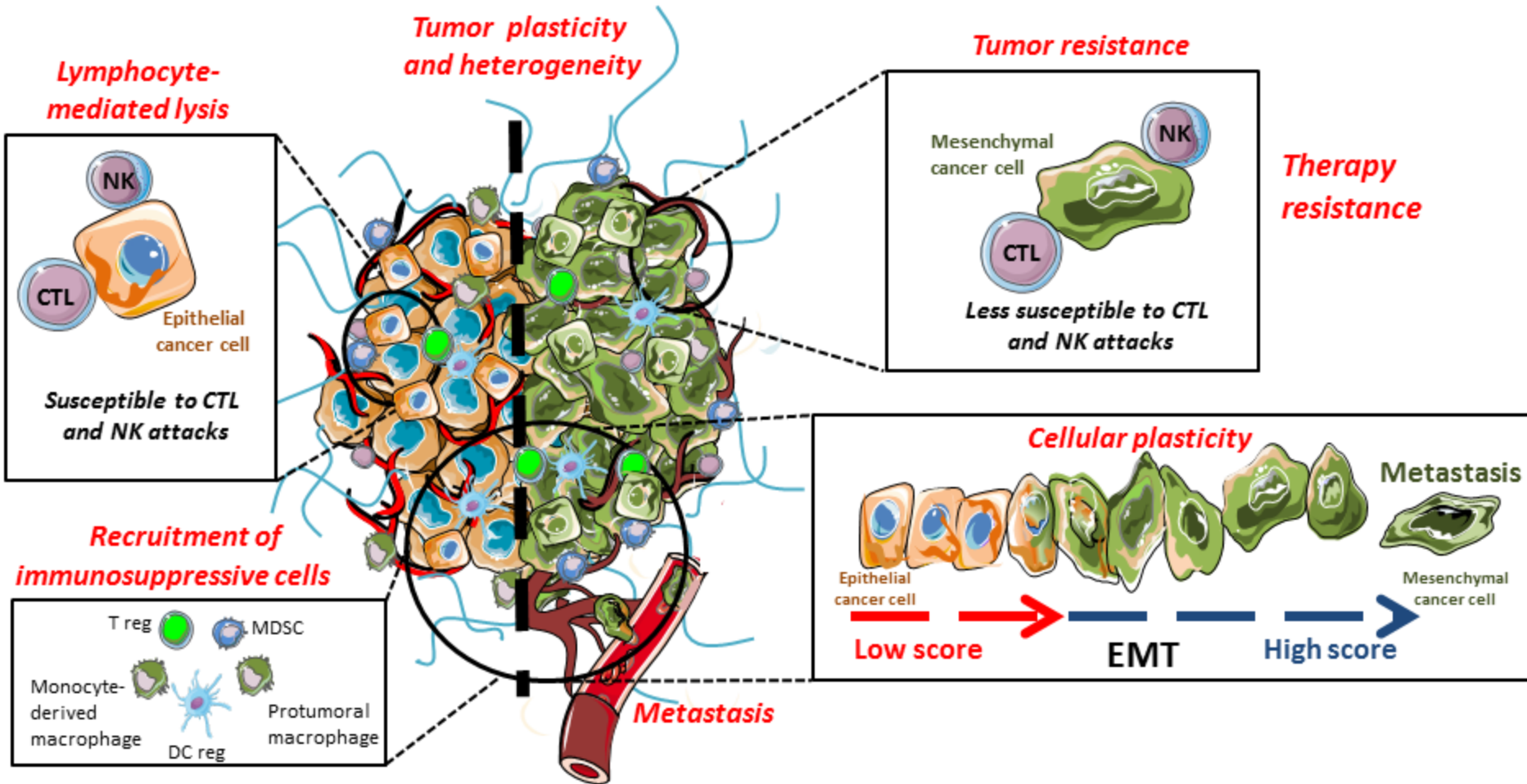
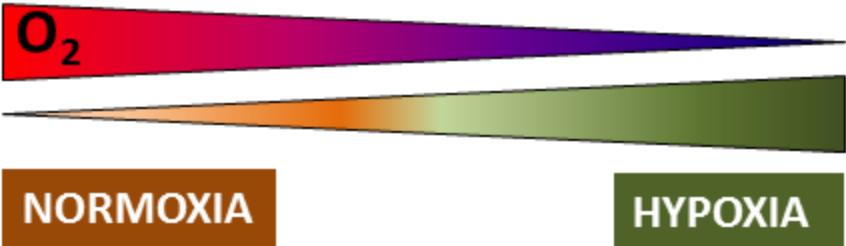


Figure 1 Schematic model for hypoxic remodeling of the tumor microenvironment in cancer. Under normal oxygen supply (normoxia), the intact tumor vasculature allows for the recruitment of immune cells which results in an increased susceptibility of cancer cells to CTL (cytotoxic T cell)- and NK (natural killer) -mediated attacks. The drop in oxygen levels (hypoxia) gives rise to a cascade of phenotypic changes resulting in increased tumor plasticity and heterogeneity. A subset of transformed cancer cells takes on mesenchymal features, reflected by a high epithelial to mesenchymal transition (EMT) score. The result is an increase in their resistance to immune cell attacks and an increase in the recruitment of immune suppressive cells.

Table 1 Prognostic hypoxia gene signatures.

Signature	Cancer cohort ^a	Prognostic end point ^b	Statistical test ^c	P-value	Point estimate ^d
253-gene epithelial hypoxia signature [27]	85 breast cancer [166]	RFS OS	Log-rank test	0.004 < 0.001	-
	72 ovarian cancer	RFS OS	Log-rank test	0.008 0.003	-
	295 breast cancer NKI dataset [167]	RFS OS	Cox PH Multivariate analysis	0.003 0.004	HR 2.38 (95% CI 1.35-4.19) HR 2.16 (95% CI 1.28-3.64)
	286 breast cancer [31, 168]	RFS	Cox PH Multivariate analysis	0.024	HR 2.20 (95% CI 1.11-4.34)
	130 lung cancer GSE4573 [31, 169]	OS	Cox PH Multivariate analysis	0.010	HR 3.15 (95% CI 1.32-7.54)
15-gene early hypoxia signature [170]	251 breast cancer GSE3494	DSS	Log-rank tests	0.005*	-
	88 neuroblastoma GSE17714 [171]	OS	Cox PH Multivariate analysis	0.012	HR 6.35 (95% CI 1.50-27.03)
99-gene hypoxia upregulated signature [28]	295 breast cancer NKI dataset [167]	MFS OS	Cox PH Multivariate analysis	0.003 0.002	HR 1.38 (95% CI 1.11-1.70) HR 1.43 (95% CI 1.14-1.81)
	60 head and neck squamous cell carcinoma GSE686 [172]	RFS	Cox PH Multivariate analysis	0.012	HR 1.96 (95% CI 1.16-3.33)
	86 Lung cancer [31, 173]	OS	Cox PH Multivariate analysis	0.021	HR 6.90 (95% CI 1.34-35.60)
13-gene VEGF profile [29]	146 breast cancer GSE3521	RFS	Log-rank test	< 0.001	-
	295 breast cancer NKI dataset [167]	RFS	Cox PH Multivariate analysis	0.001	HR 1.60 (95% CI 1.19-2.14)
	111 lung cancer [174]	Survival	Log-rank test	< 0.001	-
	50 glioma [175]	Survival	Log-rank test	0.009	-
51-gene common hypoxia metagene [31]	295 breast cancer NKI dataset [167]	MFS	Cox PH Multivariate analysis	0.002	HR 4.15 (95% CI 1.73-9.96)
	86 lung cancer [173]	OS	Cox PH Multivariate analysis	0.014	HR 12.84 (95% CI 1.71-96.50)

	130 lung cancer GSE4573 [169]	OS	Cox PH Multivariate analysis	0.023	HR 2.75 (95% CI 1.15-6.56)
7-gene chronic hypoxia signature [176]	135 hepatocellular carcinoma GSE1898 [177] and GSE4024 [178]	OS Early recurrence	Cox PH Multivariate analysis	0.020 0.008	HR 2.57 (95% CI 1.14-5.76) HR 2.90 (95% CI 1.32-6.36)
32-gene neuroblastoma hypoxia signature [171]	88 neuroblastoma GSE17714	OS	Cox PH Multivariate analysis	0.007	HR 2.73 (95% CI 1.31-5.69)
	326 head and neck squamous cell carcinoma DAHANCA 5 trial dataset [179]	LRF	Cox PH Multivariate analysis	NA	HR 1.41 (95% CI 1.03-1.94)
15-gene hypoxia classifier [30]	110 soft tissue sarcoma [161]	DSS	Bootstrapping method with 1000 iterations	0.044	HR 3.18 (95% CI 1.00-9.80)
	183 soft tissue sarcoma GSE21050 FSG dataset [180]	DMFS	Cox PH Multivariate analysis	0.017	HR 1.80
	408 soft tissue sarcoma TCGA dataset [180, 181]	DMFS	Cox PH Multivariate analysis	0.030	HR 1.72
31-gene DCE-MRI hypoxia signature [162]	155 cervical cancer GSE36562	PFS LRC	Cox PH Multivariate analysis	0.006 0.026	RR 2.50 (95% CI 1.29-4.84) RR 3.71 (95% CI 1.17-11.8)
32-gene Pimonidazole signature [163]	267 prostate cancer GSE16560 [182]	survival	Cox PH Multivariate analysis	< 0.001	RR 3.17 (95% CI 1.45-6.93)
6-gene DCE-MRI classifier [164]	281 uterine cervical cancer GSE72723	PFS	Cox PH Multivariate analysis	0.002	HR 2.12 (95% CI 1.33-3.40)
		LRC		0.012	HR 2.62 (95% CI 1.23-5.58)
		DSS		0.006	HR 2.12 (95% CI 1.24-3.61)
10-gene hypoxia responsive signature	88 melanoma TCGA dataset [184]	DFS	Logistic regression analysis	<0.001	-

[183]					
21-gene hypoxic volume signature [165]	300 oropharyngeal squamous cell carcinoma GSE65858 [185]	PFS	Cox PH Univariate analysis	0.047	HR 1.50 (95% CI 1.00-2.20)
24-gene bladder cancer hypoxia signature [33]	75 bladder cancer BCON radiotherapy arm dataset [186]	LPFS	Cox PH Multivariate analysis	0.017	HR 2.25 (95% CI 1.16-4.39)
	14 bladder cancer GSE13507	OS	Log-rank test	0.002	-
	308 urothelial cancer GSE32894 [187]	OS	Log-rank test	< 0.001	-
	80 bladder cancer GSE1827 [188]	OS	Log-rank test	0.015	-
	144 bladder cancer GSE19915 [189]	OS	Log-rank test	0.074	-
24-gene soft tissue sarcoma-specific hypoxia signature [180]	183 soft tissue sarcoma GSE21050 FSG dataset	DMFS	Cox PH Multivariate analysis	0.005	HR 2.16 (95% CI 1.25-3.70)
	127 soft tissue sarcoma GSE21050 FSG dataset	DMFS	Cox PH Multivariate analysis	0.001	HR 3.06 (95% CI 1.51-6.19)
	408 soft tissue sarcoma TCGA dataset [181]	DMFS	Cox PH Multivariate analysis	0.009	HR 2.05 (95% CI 1.19-3.53)
28-gene localized prostate cancer hypoxia signature [34]	491 prostate cancer TCGA dataset [190]	BCR free survival	Log rank test	< 0.001	-
	131 prostate cancer GSE21032 [191]	BCR free survival	Log rank test Cox PH Multivariate analysis	0.002 0.021	HR 4.59 (95% CI 1.71-12.32) HR 3.51 (95% CI 1.21-10.15)
	100 prostate cancer GSE54460 [192]	BCR free survival	Log rank test Cox PH Multivariate analysis	0.009 0.048	HR 2.12 (95% CI 1.20-3.74) HR 1.84 (95% CI 1.00-3.39)
	111 prostate cancer Cambridge dataset [193, 194]	BCR free survival	Log rank test	0.060	HR 2.54 (95% CI 0.96-6.69)
	232 prostate cancer GSE62116 DecipherGRID™ prostate cancer database (NCT02609269)	BCR free survival	Log rank test	0.007	HR 2.04
	48 prostate cancer GSE41408 DecipherGRID™ prostate cancer database (NCT02609269)	BCR free survival	Log rank test	0.002	OR 7.60

	182 prostate cancer GSE62667 DecipherGRID™ prostate cancer database (NCT02609269)	BCR free survival	Log rank test	< 0.001	OR 5.90
	212 prostate cancer CPC-GENE dataset [195]	BCR free survival	Log rank test Cox PH Multivariate analysis	0.026 0.021	HR 1.80 HR 1.81 (95% CI 1.02-3.21)
	130 prostate GSE72291 DecipherGRID™ prostate cancer database (NCT02609269)	BCR free survival	Log rank test Cox PH Multivariate analysis	0.007 0.014	HR 2.81 (95% CI 1.33-6.00) HR 2.17 (95% CI 1.17-4.01)
	248 prostate cancer Belfast dataset [196]	BCR free survival	Log rank test	0.035	-
	631 pooled prostate cancer	DMFS	Cox PH Multivariate analysis	0.003	HR 2.57 (95% CI 1.38-4.77)
42-gene basal like breast tumors hypoxia signature [197]	basal subtype breast Metabric 2012 dataset [198]	OS	Log rank test	0.031	-
	basal subtype breast Metabric 2016 dataset [199]	OS	Log rank test	0.003	-
	basal subtype breast KMplotter 2017 update dataset [200]	OS	Log rank test	0.022	-
5-gene glioma hypoxia signature [201]	395 IDH1 ^{mut} lower grade glioma TCGA dataset [202]	OS	Cox PH Multivariate analysis	< 0.001	HR 5.85 (95% CI 2.89-11.83)
	120 IDH1 ^{mut} lower grade glioma Rembrandt database	OS	Cox PH Multivariate analysis	< 0.001	HR 5.25 (95% CI 0.64-1.59)

^a Cohorts proving the prognostic capacity of each signature and reported accession numbers for datasets in gene expression omnibus are indicated (NKI: Netherlands Cancer Institute; DAHANCA 5: The Danish Head and Neck Cancer; FSG: French sarcoma group; TCGA: The Cancer Genome Atlas; BCON: bladder carbogen nicotinamide; CPC-GENE: Canadian Prostate Cancer Genome Network; IDH1^{mut}: Isocitrate Dehydrogenase 1 mutant)

^b Prognostic endpoints varied based on follow-up period and data availability (OS: overall survival; RFS: recurrence free survival; DSS: disease specific survival; MFS: metastasis free survival; LRF: locoregional failure; DMFS: distant metastasis free survival; PFS: progression free survival; LRC: locoregional control; DFS: disease free survival; LPFS: local progression free survival; BCR free survival: biochemical recurrence free survival)

^c Multivariate analysis included other clinical variables such as tumor grade, size and lymph node status, depending on the study (PH: proportional hazard)

^d Reported Hazard Ratio (HR), Relative Risk (RR) or Odds Ratio (OR) are for high hypoxic tumors vs low hypoxic tumors (CI: confidence interval)

* Mean p-value at 5 and 10 years

NA: Not Available

Table 2: Hypoxia-targeting and hypoxia-alleviating drugs in clinical trials with immunotherapeutic interventions

Drug	Inhibitory mechanisms	Mode of action	FDA^a	Clinical trial (n)^b
Hypoxia-Targeted				
Daunorubicin	DNA binding	Inhibits binding of HIF-1 to the HRE sequence	Yes	14
Doxorubicin	DNA binding	Inhibits binding of HIF-1 to the HRE sequence	Yes	71
Evofosfamide (TH-302)	Drug delivery - Hypoxia-Activated prodrugs	Upon activation in oxygen deficient zones, TH-302 is converted selectively to the drug's active form, dibromo isophosphoramidate mustard, a potent alkylator	No	1
Bortezomib	HIF-1 Transcriptional Activity	Inhibits the 26S proteasome, a large protease complex that degrades ubiquitinated proteins.	Yes	18
FK228 (romidepsin)	HIF-1 α Expression	Inhibits hypoxia-induced angiogenesis and depletes several heat shock protein 90	Yes	2
Everolimus	HIF-1 α Translation/HIF1 Expression	mTOR inhibitor that binds with high affinity to FKBP-12, thereby forming a drug complex that inhibits the activation of mTOR	Yes	12
Temsirolimus (Torisel[®]- CCI-779)	HIF-1 α Translation	Inhibitor of mTOR; Temsirolimus binds to an intracellular protein (FKBP-12), and the protein-drug complex inhibits the activity of mTOR that controls cell division	Yes	12
Topotecan	Hypoxic Signaling/HIF-1 α Translation	Inhibits the action of topoisomerase I	Yes	5
Irinotecan	Hypoxic Signaling	Inhibits the action of topoisomerase I	Yes	25
Vorinostat	Promoting HIF α degradation	Inhibits the enzymatic activity of histone deacetylases (HDAC)	Yes	8
Hypoxia Alleviating				
Bevacizumab	Angiogenesis	Inhibiting the binding of VEGF to its cell surface receptors	Yes	83
Cetuximab	Angiogenesis	Inhibits the binding of EGFR and TGF alpha	Yes	40
Sorafenib	Angiogenesis	Protein kinase inhibitor	Yes	20
Erlotinib	Angiogenesis	Inhibits intracellular phosphorylation of tyrosine kinase associated with EGFR	Yes	18

Cabozantinib	Angiogenesis	Tyrosine-kinase inhibitor	Yes	14
Rapamycin/ SIROLIMUS	Angiogenesis/ HIF-1 α Translation	mTOR inhibitor	Yes	12
Sunitinib	Angiogenesis	Targeting multiple receptor tyrosine kinases (RTKs)	Yes	10
Regorafenib	Angiogenesis	Tyrosine-kinase inhibitor	Yes	9
Gefitinib	Angiogenesis	Inhibits EGFR tyrosine kinase by binding to ATP binding site of the enzyme	Yes	8
Apatinib	Angiogenesis	Tyrosine-kinase inhibitor	Yes*	8
Panitumumab	Angiogenesis	Binds specifically to EGFR and inhibits the binding of ligands for EGFR	Yes	6
Nintedanib	Angiogenesis	Tyrosine-kinase inhibitor	Yes	6
Axitinib	Angiogenesis	VEGFR inhibitor	Yes	4
Losartan	Angiogenesis	Angiotensin system inhibitors	No	1
Brivanib	Angiogenesis	Tyrosine-kinase inhibitor	No	24
Vanucizumab	Angiogenesis	Bispecific monoclonal	No	3

^a FDA approval status for drugs targeting hypoxia-related factors

^b Number of clinical trials (n) extracted from <https://clinicaltrials.gov/>

* CFDA: Chinese FDA