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Hypoxia-inducible factor 2 α : a novel target in gliomas

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Hypoxia is an important contributor to aggressive behavior and resistance mechanisms in glioblastoma. Upregulation of hypoxia inducible transcription factors (HIFs) is the primary adaptive cellular response to a hypoxic environment. While HIF1 α has been widely studied in cancer, HIF2 α offers a potentially more specific and appealing target in glioblastoma given expression in glioma stem cells and not normal neural progenitors, activation in states of chronic hypoxia and expression that correlates with glioma patient survival. A first-in-class HIF2 α inhibitor, PT2385, is in clinical trials for renal cell carcinoma, and provides the first opportunity to therapeutically target this important pathway in glioma biology.

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Oxygen is a critical substrate for homeostatic cellular processes. Failure to regulate oxygen is detrimental to normal cell survival. Cells invest themselves in redundant and competing pathways to ensure physiologic oxygen tension. In the context of cancer, a hypoxic state dominates the tumor microenvironment due to abnormal vasculature and rapid cell proliferation [1]. The hypoxic environment triggers a cascade of malignant cellular responses which lead to tumor progression, metastases, resistance to chemoradiation and characteristics of a stem-like cell phenotype [2–7].

Glioblastoma (GBM) is a valuable model to study hypoxia in cancer. Intraoperative necrosis, radiographic-decreased oxygen uptake and histologic vascular pathology all suggest hypoxia is pathognomonic in GBMs malignant behavior [8]. Direct intraoperative measurements of oxygen tension in patients undergoing surgery for newly diagnosed malignant brain tumors demonstrated a steep hypoxic gradient across normal and intratumoral tissue. In a seminal study of patients undergoing surgery for malignant brain tumors, the mean arterial value of oxygen was 109.2 mmHg, peritumoral values of oxygen were 59.8 ± 6.5 mmHg and intratumoral values of oxygen were 15.3 ± 2.3 mmHg, providing firm evidence that hypoxia is present in brain tumors [9]. In comparison, similar measurements within the normal brain in patients under anesthesia and inhaling an FiO₂ of 100% revealed an average PO₂ of 137 mmHg [10]. In neuroimaging studies such as contrast enhanced MRI, GBMs are classically defined as ring-enhancing lesions with a central necrotic core reflective of marked hypoxia central to a tumor rapidly outgrowing its blood supply. Tumor imaging with PET (positron emission tomography) tracer fluoromisonidazole (FMISO), which binds to tissue at an oxygen tension of 2–3 mmHg and below, confirms diffuse areas of hypoxia within the majority of GBM tumors [11]. In another study using the same methodology, FMISO signal was not present in low-grade gliomas confirming hypoxia is a feature unique to high-grade tumors [12]. Furthermore, in both univariate and multivariate analyses (including age and tumor volume), increasing tumor hypoxia was associated

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with poorer patient survival [11]. GBMs demonstrate tumor hypoxia on direct measurements and indirectly on imaging studies that correlate to patient survival.

Within GBMs, microenvironments enriched for hypoxia are defined by the presence of necrosis, which can range from focal areas of micronecrosis, larger areas of geographic necrosis and/or the classic microscopic finding of pseudopalisading necrosis, where tumor cells radially extend away from a central area of necrosis. Pseudopalisading necrosis is also negatively correlated to patient outcomes [8,13,14] and represents hypoxic tumor cells migrating away from dysfunctional vasculature [8]. Gene expression in pseudopalisading cells confirms a hypoxic response with upregulation of glycolysis, angiogenesis and cell-cycle control [15]. Specific genetic changes included vascular proliferation and permeability with overexpression of *VEGF* [15], *OCT4* [16] and *RAD51* [17], which promotes vascular proliferation and permeability, resistance to chemoradiation and altered DNA repair pathway, respectively. Just as hypoxic changes are specific to high-grade gliomas on radiographic studies, low-grade gliomas lack of central necrosis on histology also lack expression of HIF2 α on immunohistochemistry analysis (small cohort from the author's unpublished work). An additional potential molecular explanation for HIF2 α downregulation in low-grade gliomas is the isocitrate dehydrogenase 1 mutation in the majority of diffuse gliomas. Isocitrate dehydrogenase mutations (R132H) appear to be linked to increased hydroxylation of HIF2 α , hypothesized to occur by accumulation of the R-enantiomer of hydroxyglutamate activating HIF-prolyl hydroxylase (PHD), thus, initiating ubiquitin tagging and degradation, and limiting hypoxic signaling in these tumors [18].

Tumor hypoxia correlates to patient survival perhaps in part due to the biological aggressiveness but also due to the impact on treatment efficacy. Hypoxia limits the effectiveness of radiation and chemotherapeutic drugs [3], which negatively impact patient prognosis [19–21], and may be one of the mechanisms driving tumor recurrence. This review summarizes the pathophysiology of hypoxia in gliomas and highlights potential targeted therapies to this pervasive environmental stress.

Hypoxia-inducible factors

HIFs were initially described in 1992 for their role in erythropoietin gene regulation [22], yet their biologic role is rooted in primitive eukaryotic adaptations to survival in hypoxic environments through activation of glycolytic pathway to meet metabolic demands [23]. The HIFs are a family of transcription factors that mediate cellular responses to hypoxia by controlling over 40 downstream hypoxia adaptive genes. HIFs bind to hypoxia response element (HRE) sequences in gene promoters, resulting in gene expression alterations. Structurally, HIFs consist of two subunits, which combine to form a heterodimer. The α -subunits (HIF1 α , HIF2 α , HIF3 α) are oxygen-responsive cytoplasmic proteins, while the β -subunit (HIF1 β , conserved) is a constitutively expressed nuclear protein. The naming convention for active heterodimer is based on the unique α -subunit, so that active HIF1 α is composed of HIF1 α and HIF1 β subunits while active HIF2 α is a heterodimer of HIF2 α and HIF1 β . The majority of the literature focuses on HIF1 α and HIF2 α as they were discovered first [24–26]. Less is known about the function and roles of HIF3 α , an isoform that antagonizes HRE gene expression [24].

In normoxic conditions (Figure 1), cells degrade cytoplasmic HIF- α subunits via hydroxylation by PHD and polyubiquitination by Von Hippel-Lindau (VHL) and E3 ubiquitin ligase. This modified α -subunit then undergoes proteasomal degradation. In hypoxia, PHD activity is reduced leading to cytoplasmic α -HIF stabilization, accumulation and translocation to the nucleus. In the nucleus, the α -subunits dimerize with a complementary β -subunit (HIF1 β , alternatively named aryl hydrocarbon receptor nuclear translocator [ARNT]). This α/β active heterodimer binds to HREs within promoter regions and drives gene expression contributing to cell proliferation, migration, metabolism, apoptosis, angiogenesis and survival [27].

HIF1 α was the first isoform identified with a function critical for cellular adaptation to low oxygen state [22,25,26,28,29]. While HIF1 α was first identified in erythropoietin regulation, [22] HIF1 α is widely expressed and critical in embryonic development, inflammatory/ischemic diseases and oncogenesis [26]. HIF2 α was identified shortly thereafter through homology searches as 48% sequence identity is shared with HIF1 α [28]. While structurally very similar, HIF1 α and HIF2 α demonstrate increasingly divergent biologic roles [5,30,31]. HIF1 α dominates the acute response to hypoxia and is ubiquitously expressed, whereas HIF2 α mediates responses to chronic hypoxia and is more selectively expressed in specific tissues [6].

The physiologic understanding of HIF signaling has expanded to include disease states with marked ischemia or inflammation [32]. More recently cancer biology has refocused on the balance of oxygen supply and consumption, hypothesizing that significant and chronic intratumoral hypoxia is the consequence of abnormal tumor vasculature,

Figure 1. In normoxia, cells degrade cytoplasmic hypoxia-inducible factor 2 α after hydroxylation by prolyl hydroxylases 1–3 and polyubiquitination by Von Hippel-Lindau and E3 ubiquitin ligase. Polyubiquitinated HIF2 α undergoes proteasomal degradation. In hypoxia, PHD activity is reduced leading to cytoplasmic HIF2 α stabilization, accumulation and translocation to the nucleus. In the nucleus, the α subunits dimerize with a complementary β subunit (HIF1 β). This α/β active heterodimer binds to HREs within DNA promoter regions and drives gene expression contributing to cell proliferation, migration, metabolism, apoptosis, angiogenesis and survival. HRE: Hypoxic response element.

Table 1. Mechanisms of hypoxia-inducible factor 2 α upregulation in tumor cells.

Event	Mechanism	Tumor type	Ref.
VHL gene mutation/deletion	Loss of proteasome degradation of HIF2 α , HIF2 α accumulates	Renal cell carcinoma	[41,44]
Hypoxia	Erratic blood supply and thrombosis of blood vessels contributing to hypoxic microenvironment	Glioblastoma	[7]
Gain of function HIF2 α mutation	Missense mutations resulting in degradation failure of HIF2 α	Paranglioma and pheochromocytoma	[45]

increased diffusion distances for cellular nutrient delivery and anemia of chronic disease [33]. Cells conditioned in hypoxia demonstrate a selective survival advantage during chemoradiation and clonally expand causing tumor progression and recurrence [34,35]. Given these observations, there is a growing interest in targeted inhibition of these maladaptive HIF pathways.

To date, inhibition of HIF1 α has demonstrated no clear clinical evidence of antitumoral activity [19–21]. Completed studies include a Phase I study with a HIF antisense mRNA (EZN-2968) in advanced carcinoma and lymphomas and an oral HIF1 α inhibitor (PX-478) in advanced solid tumors and lymphomas [36,37]. Comprehensive discussions of HIF1 α in cancer biology are referenced here [38,39]. This review focuses on the role of HIF2 α as an emerging target of therapeutic interest in gliomas.

Mechanisms of HIF2 α upregulation

To date three distinct cellular mechanisms account for upregulation of HIF2 α in an oncologic context (Table 1).

Figure 2. Immunohistochemistry for hypoxia-inducible factor 2 α performed on formalin-fixed paraffin-embedded adult glioblastoma tissue demonstrates focal positive staining in the perivascular space. This staining pattern suggests the possibility of a hypoxic gradient from dysfunctional vasculature leading to the focal upregulation of HIF2 α .

Loss of VHL gene results in failure to degrade HIF2 α

VHL is a critical protein that targets HIF2 α for proteasomal degradation. VHL is mutated in 57% or deleted (loss of heterozygosity) in 98% of renal cell carcinomas (RCCs) originally described by Gnarra *et al.* and confirmed in multiple other reports [40–44] and results in a condition termed pseudohypoxia – upregulation of hypoxic pathways in normoxia [45]. In pseudohypoxia, HIF2 α is unable to be degraded due to an inactivating mutation of the *VHL* gene. HIF2 α pairs with the constitutively expressed binding partner, ARNT, and drives expression of genes harboring HREs, thus mimicking a hypoxic environment [46]. The importance of VHL in regulation of the hypoxia pathways was demonstrated in gliomas through experiments modeling overexpression of VHL. Ectopic expression of VHL reduced the subcutaneous tumor growth rate in nude mice by 70% and was associated with reduced levels of HIF1 α , VEGF and Bcl-2 [47].

Hypoxic upregulation of HIF2 α

Hypoxia in cancer cells is classically attributed to accelerated clonal expansion and rapidly proliferating tumors that outgrow their blood supply. GBM is particularly predisposed to hypoxia due to irregular vasculature and high amounts of prothrombotic tissue factor. 50% of GBM specimens have observable microthrombi occluding vessels on routine histologic examination resulting in perivascular hypoxic nests [8,48]. Perivascular hypoxia is suggested by immunohistochemistry for HIF2 α , which reliably and reproducibly demonstrated focal perivascular clustering of HIF2 α staining (Figure 2).

At the molecular level, hypoxia shifts the balance of production and degradation resulting in upregulation of HIFs. In gliomas, the role of HIF1 α and HIF2 α is an area of active investigation. Immunohistochemical analysis of 50 glioma samples demonstrated a higher overall abundance of HIF2 α compared with HIF1 α [49]. In a TCGA (The Cancer Genome Atlas) analysis, HIF2 α is associated with elevated expression mesenchymal-associated genes on chromosome 7 [50–53]. In the REMBRANT database, HIF2 α but not HIF1 α is inversely correlated with overall patient survival [54]. Furthermore, several studies demonstrated that HIF2 α is preferentially expressed within a tumor stem cell subpopulation via CD44 and drives tumor dedifferentiation [49,54,55]. CD44 is a glycoprotein transmembrane receptor expressed on stem cells in the perivascular niche of GBMs [56]. CD44, which is transcriptionally activated by truncated glioma-associated oncogene homolog 1, can be cleaved by gamma-secretase, releasing a CD44 intracellular domain (ICD), which activates the hypoxia-response pathway [57]. Mechanistically, CD44 binds to and activates HIF2 α , and not HIF1 α , independent of oxygen levels and induces hypoxic signaling [58]. In a glioma mouse model, CD44 enhanced the hypoxic signature in perivascular tumor regions [58]. Inhibition of CD44 ICD both decreased HIF2 α expression and reduced hypoxia-induced glioma stemness, but did not impact HIF1 α expression. Thus, CD44 ICD-mediated HIF2 α expression directly promotes the hypoxic signature of gliomas, independent of HIF1 α [58]. Taken together, these studies suggest that HIF2 α is a biologically relevant protein in glioma pathology.

In several solid malignancies, HIFs mediate signaling between macrophage and mesenchymal stem cells [59], activate malignant cell motility [60] and contribute to extracellular matrix remodeling [61,62]. In hepatocellular

carcinoma, HIF2 α is associated with promoting metastases via HIF2 α -dependent expression in a hypoxic environment [63].

Gain-of-function mutations within HIF2 α

A newly identified mechanism of HIF2 α upregulation involves mutations within *HIF2 α* that prevent its degradation. Recent studies in paragangliomas and pheochromocytomas identified 40 cases with *HIF2 α* mutations, highlighting the first instance of *HIF2 α* mutation in human cancers [45,64]. The majority of mutations observed were missense mutations in the oxygen-dependent degradation domain. Hotspot mutations involving proline-531 and alanine-530 account for 62% of described *HIF2 α* mutations and result in a HIF2 α gain-of-function properties [45]. *In vivo* mouse studies of mutant HIF2 α -overexpressing HEK293T cells injected into the flank demonstrated that these gain-of-function mutations are tumorigenic [65]. This suggests that HIF2 α has the capacity to be an independent oncogenic transcription factor.

HIF2 α in GBM

In vitro studies have largely focused on the potential role that HIF2 α plays in glioma stem cell (GSC) biology given its expression in GSCs and not normal neural progenitor cells [54]. HIF2 α , not HIF1 α , is selectively upregulated in GSCs, which have been implicated in driving tumor recurrence and treatment resistance [49,54]. HIF2 α may also contribute to cell division and chemotherapy resistance. HIF2 α regulates class III, β -tubulin expression and may play a role in microtubule dynamics [66–68]. Flow cytometry analysis of temozolomide-resistant glioma cells identified HIF2 α upregulation in all chemo-resistant cell lines with increasing HIF2 α levels over time [69,70]. High levels of HIF2 α expression via immunohistochemistry were also noted in recurrent disease patient samples in our studies. These observations raise the question of whether standard-of-care glioma therapies induce HIF expression or whether increased HIF expression is a marker of recurrent tumor.

Functional studies tie HIF2 α activity to increasing the malignant phenotype. Knockdown of HIF2 α using shRNA in GSC reduced tumorsphere formation, GSC-mediated angiogenesis *in vitro* and increased glioma stem-like cell apoptosis [54,69]. Mao *et al.* confirmed HIF2 α knockdown downregulated GSC oncogenes, namely zinc finger protein 217 [71]. *In vivo* evidence supporting the biologic significance of HIF2 α targeting was demonstrated in glioma xenograft models, wherein HIF2 α knockdown delayed onset of neurologic deficits and increased survival to 158 days compared with 47 days in controls [54]. Increased HIF2 α expression is observed to be present in high-grade, aggressive and therapy-resistant gliomas and reducing HIF2 α expression experimentally mitigates the malignant cellular phenotype and increases animal survival.

Data supporting the biologic importance of HIF2 α in patients are largely retrospective. HIF2 α expression, not HIF1 α , was linked to patient survival using the REMBRANDT database (n = 834) [54]. In a prospective clinical trial testing bevacizumab/irinotecan in GBM patients, carbonic anhydrase 9 (CA9) and HIF2 α expression were major and significant predictors of treatment effectiveness and overall survival [72]. Patients with high CA9 and HIF2 α expression had the worst survival outcomes, whereas patients with negative CA9 and negative HIF2 α had the best survival outcomes [72]. These genetic studies suggest that HIF2 α expression may be a target of therapeutic significance.

Therapeutic implications

Targeting transcription factors in oncology has largely been overshadowed by the development of small-molecule-targeted agents against downstream targets. Within the hypoxia pathway, a VEGF-A inhibitor, bevacizumab (Avastin) gained FDA approval for colorectal cancer in 2004 [73] and accelerated approval in 2009 followed by full approval in 2017 for recurrent GBM. Since this time, numerous drugs targeting VEGF including antibodies and receptor tyrosine kinase inhibitors have been developed including sunitinib, axitinib, sorafenib and lenvatinib [74]. Unfortunately, sustained responses to these agents have not been realized in the clinical setting, refocusing interest in transcription factor inhibition. Indeed, large drug library screens have identified potential HIF inhibitors [75,76]. Clinical development of these compounds, which include echinomycin and cardiac glycosides [77], is limited by a lack of specificity of the inhibitors. Preclinical agents with nonspecific inhibition of HIF1 α have been tested in GBM without significant results including bortezomib [78], echinomycin [76], amphotericin B [79] and 2-methoxyestradiol [80]. With an improved understanding of the potential biologic significance of HIF2 α , drugs targeting this transcription factor were anticipated for further development.

Identifying a specific molecule to target HIF2 α began in the early 2000s with magnetic resonance spectroscopy and crystallography to define the HIF2 α /ARNT-binding pocket, a configuration not formed by HIF1 α or

HIF3 α [81,82]. An extensive small-molecule screen identified 130 potential compounds of which ultimately resulted in the development of PT2385, a first-in-class HIF2 α inhibitor, currently being tested in patients with clear-cell RCC by Peloton Therapeutics Inc. [83]. A Phase I dose-finding study demonstrated a favorable safety profile with anemia and fatigue being the most common treatment-emergent adverse events and determined the recommended Phase II dose (800 mg taken by mouth twice daily) [83]. The agent is orally available, has favorable blood–brain barrier penetrating properties including 90% oral bioavailability in dogs and high brain/plasma ratio (0.9) in rats [40,84].

A multi-institution Phase II study of PT2385 in patients with recurrent GBM is ongoing within the Adult Brain Tumor Consortium – ‘*Single-arm, Open-label Phase II Efficacy Study of First-in-class HIF2-Alpha Inhibitor, PT2385, for Patients with Recurrent Glioblastoma.*’ The optimal timing of treatment within the standard of care for patients with gliomas has yet to be defined. Synergy with radiation is suspected given hypoxic cells tend to be radioresistant and PT2385 may target this population.

Conclusion

Hypoxia is a driver of the malignant phenotype in GBM and understanding the resultant biology provides opportunities to develop novel therapeutic approaches to address this maladaptive environmental stress. Most targeted therapeutic strategies rely on enzymes or cell surface receptors; however, targeting transcription factors are gaining interest in oncology [85]. HIF2 α in gliomas is an attractive candidate as expression tracks with glioma malignancy in tumor location and tumor grade. As a transcription factor, HIF2 α is upstream in the signaling pathway and inhibiting its signaling should result in simultaneously downregulating multiple targets. Additionally, since HIF2 α is an upstream target, being a transcription factor, resistance mechanisms including pathway redundancies or compensatory pathways may be limited. Targeting HIF2 α may disrupt the tumor cells’ response to environmental signals. HIF2 α is a specific and druggable target in gliomas and deserves further thorough preclinical and clinical evaluation.

Future perspective

As of this writing, specific HIF2 α inhibitors are yet to be US FDA approved. Clinical trials are currently in progress and hypoxia will be a new clinically utilized therapeutic target in oncology the next 5–10 years with the FDA approval. As seen in the development of EGFR inhibitors, a library of different compounds gaining the FDA approval is anticipated. The first cancer likely to receive the FDA indication is RCC. Other solid malignancies including pancreatic and prostate cancer are likely to follow. Targeted HIF2 α inhibitors will also likely be a component of rational combinational therapy in intracranial neoplasms including GBM and paragangliomas. Patients will be assessed for hypoxia before commencing treatment to increase the likelihood of a clinical response through a combination of imaging, tumor staining and serum biomarkers. Imaging (specifically PET FMISO) will play an increasingly important role in assessing tumor hypoxia prior and during treatment as this modality is under current evaluation for predicting response to therapy and outcome. Additional biomarkers to be tested for clinical response include immunohistochemistry for CA9 and the HIFs, genetic sequencing for VHL and HIF mutations, and gene copy number assays for VHL deletion. As radiation efficacy is reduced in hypoxic states, antihypoxia therapies are likely to prove synergistic with radiotherapy and to become a component of a combinational therapy approach. The delivery of antihypoxic treatments may also evolve to locoregional rather than systemic. Resistance or nonresponders to antihypoxia treatment will also be evaluated for upregulation of escape/compensatory pathways and lead to new therapy combinations or further insights on targeting the oncogenic response. Additionally, the status of tumor initiating stem-like cells will be of interest as currently these cells are hypothesized to reside in hypoxic niches. Thus, antihypoxia therapies have the potential to have broad impact as a treatment strategy in oncology.

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Executive summary

Hypoxia-inducible factors

- Hypoxia is fundamental to the malignant biology of cancer including glioblastoma (GBM).
- Transcription factors, hypoxia-inducible factors (HIFs), mediate the hypoxic response including HIF1 α and HIF2 α .
- HIF2 α appears to be a specific and targetable protein upregulated in human cancer.

Mechanisms of HIF2 α upregulation

- HIF2 α is upregulated in cancer through multiple mechanisms including hypoxia, inactivation of HIF2 α regulators (i.e., von Hippel-Lindau) and HIF2 α genetic gain-of-function mutations.

HIF2 α in GBM

- HIF2 α is the hypoxia response transcription factor that is specific to glioma cells and not normal neural progenitors.
- HIF2 α is correlated with glioma patient survival and upregulation and downregulation directly effect tumor growth in preclinical mouse models.

Therapeutic implications

- A first-in-class HIF2 α inhibitor, PT2385, is under investigation in renal cell carcinoma and warrants investigation in GBM.

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