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# The neglected burden of chronic hypoxia on the resistance of glioblastoma multiforme to first-line therapies

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**Abstract**

Glioblastoma multiforme (GBM) is the most common adult primary brain tumor. The standard of care involves maximal surgery followed by radiotherapy and concomitant chemotherapy with temozolomide (TMZ), in addition to adjuvant TMZ. However, the recurrence rate of GBM within 1–2 years post-diagnosis is still elevated and has been attributed to the accumulation of multiple factors including the heterogeneity of GBM, genomic instability, angiogenesis, and chronic tumor hypoxia. Tumor hypoxia activates downstream signaling pathways involved in the adaptation of GBM to the newly oxygen-deprived environment, thereby contributing to the resistance and recurrence phenomena, despite the multimodal therapeutic approach used to eradicate the tumor. Therefore, in this review, we will focus on the development and implication of chronic or limited-diffusion hypoxia in tumor persistence through genetic and epigenetic modifications. Then, we will detail the hypoxia-induced activation of vital biological pathways and mechanisms that contribute to GBM resistance. Finally, we will discuss a proteomics-based approach to encourage the implication of personalized GBM treatments based on a hypoxia signature.

**Keywords** Glioblastoma multiforme, Hypoxia, HIF-1α, HIF-2α, Proteomics

## Background

Glioblastoma multiforme (GBM) is the most lethal primary adult brain cancer with a high recurrence rate. The current first-line therapy for treating newly diagnosed GBM is known as the "Stupp protocol" consisting of a surgical resection whenever possible followed by concomitant radiotherapy (RT) and chemotherapy (CT), namely temozolomide (TMZ) [1, 2]. Historically, a total dose of 60 Gy is prescribed divided over 30 sessions of 2 Gy each, in addition to the concomitant oral administration of TMZ at 75 mg/m$^2$/day over 6 weeks. Then, 1 month after the end of the initial treatment, an adjuvant chemotherapy with TMZ alone is administered at a dose of 150–200 mg/m$^2$/day over a period of 5 consecutive days/month for at least 6 months [3, 4]. Recently, additional approaches have been incorporated in the clinical management of GBM, including the concomitant use of tumor-treating fields (TTF), which is strongly recommended for patients who have completed the chemo-radiotherapy protocol [5, 6]. The concomitant administration of TMZ and TTF following chemo-radiotherapy extended the median progression-free survival (PFS) to 6.7 months versus 4 months for TMZ group [7], while other studies also revealed significant effects of TTF on PFS and the overall survival (OS) [8].

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Similarly, the hypofractionation radiotherapy (hRT) regimen, characterized by increased dose per fraction (>2 Gy/fraction) [9–11], has emerged as a possible recourse for treating elderly GBM patients (>70 years old), aiming to improve the survival-to-treatment time ratio, particularly for those unable to complete standard long-course normofractionation radiotherapy (nRT) due to health issues [12]. The efficacy of hRT compared to nRT is assessed by the treatment completion rate [13]. Nevertheless, recent studies recommend combining hRT with TMZ for newly diagnosed GBM in elderly patients with good performance status, with further stratification based on the $O^6$-methylguanine DNA methyltransferase (MGMT) promoter methylation status for those with a poorer performance [14]. Therefore, modern hRT trials include adjuvant CT with TMZ [15] on one side, adjuvant immunotherapy [16] on the other side or even particle therapy such as protons and carbon ion beams [17].

However, despite a multimodal approach and advancements in imaging techniques and RT delivery methods, such as 3D-treatment planning, intensity-modulated radiotherapy (IMRT), volumetric modulated arc therapy (VMAT), and stereotactic RT, GBM inevitably develops resistance after a short period. Although updates to the classical "Stupp protocol" have resulted in modest improvements in the PFS and OS, GBM patients continue to experience high recurrence rates, with the median OS ranging from 12 to 18 months and a relative 5-year survival rate since diagnosis below 8% regardless of the gender. One of the identified factors behind this aggressive behavior is the development of tumor hypoxia, known as chronic or limited-diffusion hypoxia.

Several preclinical and clinical studies have demonstrated the negative contribution of hypoxia on tumor progression and its implication on the negative outcome of GBM. As the distance between blood vessels and the expanding tumor increases, oxygen-deprived regions, known as hypoxic zones, emerge. In response to the absence of oxygen, GBM cells will activate hypoxia-induced factors (HIFs) to compensate for energy production, therefore triggering several downstream targets implicated in cellular metabolism, angiogenesis, invasion, proliferation, and immunosuppression among others. Yet, hypoxia is still overlooked in the clinical management of GBM, whether in its diagnosis or treatment. Therefore, in this review, we will focus on the role of tumor hypoxia in driving the resistance of GBM to first-line therapies, in addition to the hypoxia-induced alterations and hypoxia-activated biological mechanisms that render GBM extremely challenging to eliminate. In addition, we will discuss a proteomics-based approach that might help in developing a personalized treatment based on the identification of a hypoxia signature.

## Development of tumor hypoxia in GBM

Oxygen is required by cells to establish oxidative phosphorylation and maintain homeostasis. Hypoxia implies the limited availability of oxygen in a certain environment. Oxygen restriction to vital organs caused by failure of the respiratory system, low levels of hemoglobin, insufficient blood flow, or any other pathological condition leads to hypoxia in these tissues [18]. Several forms of hypoxia exist, including transient or reversible, chronic or persistent, and cyclic hypoxia [19]. The severest form is chronic hypoxia, which contributes to the evolution of several diseases such as but not limited to ischemic heart disease, congestive heart failure, pulmonary hypertension, chronic obstructive pulmonary disease, acute liver failure, liver fibrosis and cirrhosis, acute kidney injury, and chronic kidney disease [18].

In GBM, hypoxia plays an essential role in the tumor development and morphological evolution. The tissue oxygen tension ($pO_2$) in the brain differs greatly and is region specific, while consuming up to 20% of the total body's oxygen. In GBM, the $pO_2$ is lower in intratumoral regions (1.25% $O_2$) when compared to peritumoral regions (2.5% $O_2$) or normal brain tissue (3–7% $O_2$) [20, 21]. This phenomenon is the result of chronic hypoxia also referred to as diffusion-limited hypoxia, which occurs in tumor regions that experience long exposure periods to low oxygen tension due to the increased distance from near blood vessels (180 μm). Several factors contribute to the development of hypoxia within the tumor. Emerging solid tumors use the oxygen and blood supply of the host organ at first. However, due to the continuous expansion of the tumor, its metabolic demand and oxygen consumption increase while the supply remains constant in the normal tissues. Therefore, the growing tumor uses the vasculature of the host organ to develop its own vascular network, via angiogenesis among other vascular generation processes [22, 23]. The product of angiogenesis is a chaotic tumor vasculature with morphological variations as compared to an organized normal vasculature [24]. In addition, the newly formed blood vessels are primitive, structurally unstable, and highly permeable causing the formation of intra-tumoral regions with energy deprivation, low glucose, increased acidity, and oxygen deficiency [4, 22]. The chaotic and immature nature of newly formed tumor blood vessels exhibit plasma leakage due to increased vascular endothelial growth factor A (VEGF-A), persistent hyperpermeability, and an inadequate lymphatic drainage, leading therefore to fluid accumulation which increases interstitial pressure, contributes to vascular stasis and the formation of necrotic regions [25, 26]. Notably, radiological analysis in GBM patients preceding GBM recurrence revealed that hypoxia could represent

a potential biomarker of recurrence. GBM exploits the existing blood vessels to infiltrate normal tissue in a process known as vessel cooption. For instance, GBM cells arrange themselves around microvessels accompanied by an increase in angiopoietin-2 (ANG-2) leading to vessel regression and thus a decreased $pO_2$. This phenomenon has been detected in recurrence regions leading to decreased vessel perfusion and increased hypoxia. Even though vessel perfusion increased again later due to neoangiogenesis, the detected hypoxia intensified and peaked at 90 days pre-recurrence, revealing the contribution of hypoxia in the malignancy of GBM [27].

The development of tumor hypoxia results in drastic events on GBM development inducing several genetic and epigenetic modulations, in addition to the hypoxia-induced alteration of vital biological pathways that contribute to the progression, treatment resistance, and GBM recurrence.

### Hypoxia-induced genetic and epigenetic alterations in GBM

Like most cancers, GBM shares a set of common genetic mutations with solid tumors. For example, *TP53* and *PTEN* mutated genes are frequently identified in GBM but are not considered as prognostic markers [28]. In addition, GBM is well known for being a heterogeneous tumor, with both intra- and inter-tumoral heterogeneity. Historically, four subtypes of GBM were identified based on genomic analysis of The Cancer Genome Atlas (TCGA), such as classical, proneural, neural, and mesenchymal. The classical subtype is the most responsive to conventional treatments, and the isocitrate dehydrogenase (IDH) mutation is commonly observed in the proneural subtype, while an overactive transforming growth factor β (TGF-β) signaling distinguishes the mesenchymal subtype and is linked to therapy resistance and early tumor recurrence [29]. Meanwhile, the neural subtype signature revealed the presence of normal neurons within the tumor, thus reflecting the infiltrative capacity of GBM. This high migration capacity manifested by the high infiltration rate into the surrounding healthy tissue renders a total surgical resection extremely challenging. Even when coupled to RT and CT, the eradication of GBM is not fully achieved. Other classifications of GBM based on transcriptomic or proteomic analyses have been proposed. For instance, two different proteomics clusters were identified in *IDH*-wild type GBM. One cluster exhibited higher stem-cell markers and a poor prognostic marker FKBP prolyl isomerase 9 (FKBP9), while the second cluster showed markers of differentiated oligodendrocyte and astrocyte with a phosphoglycerate dehydrogenase (*PHGDH*) serving as a favorable prognostic biomarker [30]. Similarly, other studies have proposed additional pathways in the classification of *IDH* status in GBM. Notably, the WNT signaling pathway has been shown to be different between *IDH*-wt versus *IDH*-mut GBM, with the WNT/β-catenin pathway being upregulated in hypoxic regions of *IDH*-wt GBM, thus promoting vessel development and cell proliferation [31]. In addition, most of the recurrent GBM is detected within a proximity to the resected tissue margin (~2–3 cm) [32]. Therefore, recent studies have focused on characterizing the peritumoral region that might partially explain the high resistance and recurrence rates in GBM due to its enrichment in GBM stem-like cells (GSCs). In addition, GBM is also famous for its rapid growth and highly proliferative capacity known as hypercellularity. Regions of cellular tumor, infiltrating tumor, microvascular proliferation, and palisading cells around necrosis are all pathological hallmarks of GBM [33]. Due to the invasive capacity of GBM and its continuous expansion, tumor hypoxia will develop with the increasing distance from normal blood vessels, and the demand for oxygen supply will certainly increase forcing the tumor to develop its own vascular system through angiogenesis. Therefore, one important focal aberration in protein coding sequences that regulates angiogenesis include the epidermal growth factor receptor (*EGFR)* amplification [34]. EGFR, one of the four members of the receptor tyrosine kinase family, phosphorylates a set of downstream effectors linked to cellular division and survival [35]. Activated EGFR and constitutively active EGFRvIII promote GBM cell proliferation and survival through the activation of the PI3K/Akt and JAK/STAT signaling pathways. Distinct oncogenic pathways downstream EGFR and EGFRvIII, such as Src, c-Myc, and nuclear factor kappa B (NFkB), are activated as well to initiate and maintain angiogenesis [34]. In addition, VEGFA upregulation was highly correlated with *EGFR* gene mutations and control angiogenesis or microvascular proliferation. Neoangiogenesis, which relies on the overexpression of nestin and the involvement of pericytes, aims to nourish the developing tumor and compensates for oxygen deficiency in hypoxic regions [32]. Pro-angiogenic growth factors including VEGF, TGF-β, fibroblast growth factors (FGFs), angiopoietin-1 (Ang-1), and epidermal growth factor (EGF), once activated bind to their specific receptors on endothelial cells and induce the degradation of the basement membrane and extracellular matrix (ECM) [36]. Next, matrix metalloproteinase (MMPs) such as MMP-2 and MMP-9, will cooperate with stromal cells to generate a new matrix. As such, endothelial cells will migrate and proliferate within this matrix, giving rise to endothelial tube-like structures that are later on surrounded by mature vascular basement membrane, pericytes, and smooth muscle cells to stabilize the newly

formed vessels [36, 37]. Interestingly, a correlation has been traced between the microvascular density of newly formed vessels at distant regions from the GBM bulk and the median survival of patients [38].

Notably, the most important genetic mutation that is required for GBM taxonomy and diagnosis is the *IDH1* or *2*. Heterozygous mutations to the *IDH* gene reduces the normal activity level of these enzymes to almost the half, as a result, isocitrate is converted into α-KG by the wild-type monomer and D-2-hydroxyglutarate (D-2-HG) by the mutated one, which accumulate in the cytoplasm and disrupt the redox homeostasis [39]. IDH mutations are frequently detected in secondary GBM, which usually develops from low-grade gliomas (LGG), while *IDH*-wt is linked to primary GBM [40]. GBM with *IDH1/2* mutations have improved prognosis and better survival; however, these represent less than 10% of GBM cases [41]. Given the critical role of *IDH* in GBM, the use of mutant selective small molecule *IDH* inhibitors such as IDH305 has been investigated in phase I clinical trials. However, targeting *IDH* remains challenging due to the different phenotypes of *IDH*-mutant cells with the different types of alterations that arise during tumor evolution. Although these inhibitors effectively reduce the accumulation of 2-HG, their impact on the growth of glioma cells has shown contradictory results [42]. It is noteworthy to mention that a correlation between hypoxia and the oncometabolite 2-HG accumulation was established. Interestingly, the product of the mutant IDH, D-2-HG, decreases the activation of hypoxia transcription factors, which might partially explain the reason behind the improved outcome of *IDH*-mt GBM versus *IDH*-wt [42, 43]. Another molecular change that takes part in the detailed classification and characterization of GBM is the *TERT* promoter mutation. Studies have shown that the knockdown of GABPB1L complex that binds the mutant *TERT* and induces its reactivation reduced the growth of GBM and sensitized the latter to TMZ [44]. Similarly, the DNA repair protein MGMT is equivalently important as a predictive marker of GBM response to CT [32]. The epigenetic silencing of *MGMT* gene promoter region by methylation has been linked to an enhanced outcome determined by a longer survival in patients treated with alkylating agents, namely TMZ, compared to patients with no promoter methylation [45]. Noteworthy, both hypoxia inducible factors (HIFs), HIF-1α and HIF-2α, bind directly on the promoter region of MGMT under hypoxia, providing evidence of the hypoxia-induced expression of MGMT and its affiliated resistance to TMZ when its promoter region is hypomethylated [46].

To emphasize further on the role of hypoxia in GBM, a list of 14 genes directly associated with hypoxia has been identified using data from the TCGA of GBM cohorts. Univariate and multivariate regression analyses were performed to develop a prognostic risk-scoring model based on the identified genes. The identified prognostic genes that were used to develop this scoring system were as follows: adrenomedullin (*ADM*), caveolin 1 (*CAV1*), prohibitin 2 (*PHB2*), procollagen-lysine,2-oxoglutarate 5-dioxygenase 2 (*PLOD2*), HSPB1-associated protein 1 (*HSPBAP1*), proteasome 26S subunit, ATPase 2 (*PSMC2*), histone-lysine N-methyltransferase (*SUV39H2*), matrix metallopeptidase 14 (*MMP14*), promyelocytic leukemia protein (*PML*), p21 activated kinase 1 (*PAK1*), matrix metallopeptidase 2 (*MMP2*), solute carrier family 9 member A1 (*SLC9A1*), cytoglobin (*CYGB*), and aquaporin 3 (*AQP3*). As a result, patients were divided into two groups of high-risk versus low-risk, with the high-risk group having a significantly lower OS than that with the low-risk score, highlighting the negative contribution of hypoxia on GBM outcome [47]. Finally, there are increasing efforts to include pre-treatment imaging techniques, such as magnetic resonance imaging (MRI) via BOLD MRI or positron emission tomography (PET), to detect the extent of tumor hypoxia as non-invasive prognostic tools in the process of personalized protocols. For instance, the application of PET scans with 18F-FMISO is a proficient method for quantifying tumor hypoxia in GBM, revealing different patterns of hypoxia and necrosis depending on the used parameters [48]. Similarly, using radiomic features from MRI along with the application of a hypoxia enrichment score generated from a set of 21 genes that are directly implicated in the hypoxia signaling pathway, authors were able to distinguish patients of short-, mid-, and long-term survival [49]. Advanced imaging techniques have the potential to accurately detect tumor hypoxia during the diagnostic evaluation of GBM enabling improved classification of GBM in responder versus non-responder groups.

### Hypoxia-induced aggressiveness and resistance of GBM to first-line therapies

Major limitations to conventional protocols and GBM therapies include the blood brain barrier (BBB) penetration, immunosuppressive tumor microenvironment, surgical resection of infiltrative GBM cells, and cognitive sparing strategies during irradiation among others. Because of the infiltrative and diffuse nature of GBM, complete surgical resection is impractical. In addition, the accessibility and extent of surgical resection depends on tumor size and location in the brain, where tumors in the brainstem or diencephalon have a low accessibility as compared to other brain regions [50]. Patients with GBM tumors in the white matter of the temporal-parietal junction had a significant decline in the Karnofsky performance status (KFS) post-surgery and were associated

with a shorter PFS and OS [51]. Therefore, treating GBM using conventional therapies including RT and CT post-surgery is inevitable to enhance the OS of patients, even if the resistance phenomenon is mostly certain.

Molecular mechanisms and pathways governing radio- and chemoresistance are still under investigation with a particular interest and emphasis on the role of hypoxia in driving the acquired resistance. Tumor hypoxia has been shown to regulate a vast majority of malignant pathways involved in GBM progression and survival mediated by the activated HIFs. In fact, higher mRNA expression and protein levels of hypoxia transcription factor, HIF-1α, were linked to the severity of GBM and its poor prognosis [52]. Activated HIFs under hypoxic conditions increase the transcription of several target genes such as VEGF, which drives angiogenesis, rendering GBM a highly vascularized tumor. However, this neovascularization system is often disrupted, because of the presence of weak junctions that link endothelial cells in addition to the absence of pericytes and smooth muscle cells required to stabilize the newly formed vessels. Consequently, the increased permeability of these vessels results in their frequent rupture and reduces the bioavailability of drugs delivered into the tumor, therefore causing an increased resistance rate [52]. Moreover, the structural misdistribution of these newly formed blood vessels leads to necrosis due to the uneven oxygen diffusion within the tumor. Consequently, hypoxia increases in ruptured regions causing the initiation of hypoxia-induced signaling and activation of the consequent target genes resulting in more GBM aggressiveness manifested by the tumor's resistance to standard therapies [52]. Therapies targeting EGFR mutations were developed including small-molecule tyrosine kinase inhibitors (erlotinib, gefitinib, afatinib) and CAR-T cell approaches among others. However, similarly to other strategies, GBM tend to resist and results have been discouraging so far. Alteration in the expression of the EGFR inhibitor targets, i.e., target independence, or induced-activation of compensatory pathways, i.e., target compensation, might explain the reasons behind GBM resistance to such therapies [53]. Furthermore, hypoxia controls the highly suppressive immune microenvironment surrounding GBM, even though macrophages and microglia constitute 30–50% of the tumor mass [32]. As a result, activated macrophages and microglia in GBM proximity are highly correlated with poor prognosis due to the immunosuppression mediated by pro-inflammatory molecules (TNF, IL-6, IL-12, and IL-β), in addition to the induction of T helper 1 (Th) cells by GBM-associated microglia leading to a lower tumor response and higher resistance [32]. Furthermore, hypoxia regulates cell cycle, apoptosis, autophagy, and aerobic glycolysis all of which promote tumor survival, invasiveness, and resistance [4]. For instance, hypoxia transcription factors enhance the expression of stem cell genes causing the persistence of GBM stem-like phenotype, increasing heterogeneity within the tumor, and inducing a subsequent chemo- and radioresistance [52].

Besides, hypoxia plays a key role in triggering radioresistance by reducing the levels of reactive oxygen species (ROS) due to the increased expression of PDK1. The formation of ROS during radiation increases the amount of secondary DNA damage caused by ionizing radiation, and if the oxygen concentration at a given irradiation time is reduced, cells become resistant to the radiation-induced DNA damage at conventional doses [22, 54]. Furthermore, hypoxia has been associated with the increased expression of the left–right determination factor (LEFTY) and Nodal, members of the TGF-β family contributing to an epithelial-to-mesenchymal transition (EMT), cell survival, and resistance under such conditions [55]. In addition, hypoxia promotes invasiveness in GBM through the upregulation of MMP-2 and −9, ZEB1, and Twist1 on invasive ends of GBM cells, to enhance the EMT transition by downregulating E-cadherin and upregulating N-cadherin [56]. Finally, under hypoxic conditions, the GBM stem-like profile is maintained through the upregulation of PI3K/Akt, JAK/STAT, and Wnt/β-catenin pathways among others to promote self-renewal, EMT, and colony formation of GSCs, leading to the anticipated treatment resistance and recurrence [57]. Likewise, several protein expression was found to be upregulated under hypoxia which contributed to shorter patient survival rates, such as monocarboxylate transporter-4 (MCT4), protein phosphatase 2A (PP2A), Kruppel-like 4 (*Klf4*), and ATP-binding cassette B1 (ABCB1) [57].

Identifying the molecular pathways regulated by hypoxia will increase the potential for identifying novel therapeutic combinations to better target the hypoxic niche in GBM.

### Hypoxia-induced activation of the biological mechanisms contributing to the resistance of GBM

The prevalent tumor and microenvironment hypoxia in GBM activates HIF-1α and HIF-2α, thereby controlling tumor survival and progression through various biological mechanisms. For instance, hypoxia regulates angiogenesis, metabolic adaptation and reprogramming, cell invasion, and survival, giving rise to the poor prognosis of GBM patients [52]. In addition, hypoxia contributes to the activation of several signaling pathways which accelerate tumor malignancy and resistance to conventional treatments, such as TGF-β, Wnt, SHH, Notch, and transcriptional factors like SNAIL1, SLUG, TWIST, and ZEB1 [58]. In the following section, we will focus on

the most common hypoxia-induced biological mechanisms in GBM that are activated under chronic or diffusion-limited hypoxia by HIF-1α and HIF-2α including angiogenesis, metabolic shift, autophagy, maintenance of stem-like phenotype, reduction of ROS, and immunosuppression, all hallmarks of GBM aggressiveness (Fig. 1).

### *Angiogenesis*

To overcome the decreased supply of oxygen and nutrients in hypoxic and necrotic regions, GBM forms an invasive front to evade normoxic regions outside the tumor forming hypercellular zones that surround necrotic areas known as the pseudopalisades [36, 52]. Microvascular hyperplasia is a characteristic angiogenic feature in GBM, manifested through tufts of endothelial cells, pericytes, and smooth muscle cells, at the leading borders of newly formed vessels. The interaction between cancerous cells of the hypoxic tumor with the surrounding normoxic tissue leads to the secretion of proangiogenic factors, such as VEGF and Il-8, to form new vessels [36]. However, the end-result of angiogenesis is the generation of defective and permeable blood vessels that may rupture at any given time leading to the formation of hypoxic foci, another landmark of GBM. Proangiogenic factors bind to their receptors on endothelial cells of old vessels causing their degradation. As a result, this will alter the extracellular matrix and the endothelial cell basement membrane in an attempt to break ground for newly formed vessels that are required for nutrient and oxygen supply [52]. The expression level of two angiogenic key regulators, VEGF-A and angiopoeitin-2 (ANGPT2), regulates the degree of neovascularization. *VEGF* is a direct target gene of HIF-1α and HIF-2α, which upon expression binds to its receptor VEGFR and initiates a series of signaling cascades to drive angiogenesis. Similarly, placenta-like growth factor (*PlGF*), platelet-derived growth factor (*PDGFB*), stromal cell-derived factor-1 (*SDF-1*), stem cell factor (*SCF*), angiopoietin

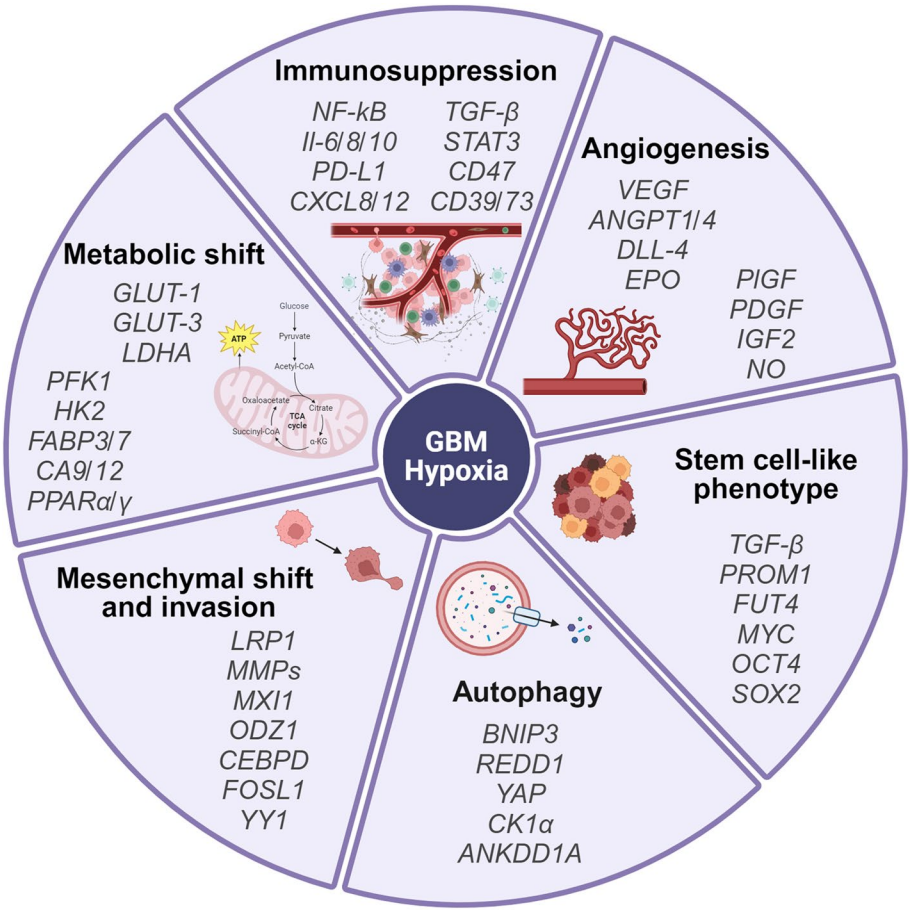


**Fig. 1** Hypoxia-induced genes implicated in vital biological processes contributing to GBM resistance to first-line therapies. Tumor hypoxia drives the resistance of GBM through the activation of genes and increased protein expression linked to the survival and maintenance of cancer cells under chronic hypoxia. Cellular and molecular adaptations driven by hypoxia are responsible for the aggressive nature of GBM to escape cellular death and manifest treatment resistance (created in BioRender)

(*ANGPT1* and *2*), and erythropoietin (*EPO*) are all target genes of HIF proteins and participate in the intricate process of angiogenesis [52, 58]. Accordingly, HIF-1α stimulates vessel growth as HIF-2α boosts vessel maturation [58]. Furthermore, HIF-1α promotes the upregulation of PDGF proteins, which in turn activates PI3K/AKT and MAPK/RAS oncogenic pathways rendering the tumor more aggressive [52]. Recently, studies have shown that RhoJ, which is highly expressed in endothelial cells, contributes to the regulation of angiogenesis via the JNK/VEGFR2-PAK-ERK signaling pathway [59]. Despite the fact that aberrant angiogenesis is frequent in GBM, some factors tend to negatively regulate HIF genes to decrease the aforementioned phenomenon. For instance, the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) has been linked to a decreased survival rate among GBM patients due to its role in inhibiting the AKT signaling pathway, leading to a reduced *VEGF* expression and counteracting the EMT observed in GBM [60].

#### *Metabolic shift to aerobic glycolysis and redox regulation*

In spite of hypoxia and the lack of oxygen, tumor cells would survive better instead of limiting their progression. This significant role is mainly attributed to HIF-1α driving the metabolic shift to glycolysis under anaerobic conditions and the consequent modulation of the lipid metabolism by HIF-2α to enhance tumor growth. Normally, cells use the mitochondrial aerobic respiratory chain to produce energy in the form of ATP through the oxidative phosphorylation of glucose and the incorporation of pyruvate in the citric acid cycle (TCA). Conversely, in cancer cells, aerobic glycolysis is a common energy production source whether oxygen is present (Warburg effect) or not. For instance, glucose is not converted into pyruvate but instead to lactate via the upregulated glycolytic enzymes in the cytosol. The end-result of aerobic glycolysis is the generation of two ATP molecules versus 38 ATP molecules produced via mitochondrial oxidative phosphorylation. Nonetheless, the kinetics of aerobic glycolysis and hence the generation of lactate from glucose is much faster than the oxidative phosphorylation of glucose in the mitochondria. Therefore, the energy produced over a defined period of time under both types of glucose metabolism is comparable [61]. This compensation in kinetics could be attributed to the stabilized HIF-1α under hypoxia, which promotes the transcription of glucose transporters namely GLUT1 and GLUT3, to increase glucose uptake into the cancer cells [52]. In addition, HIF-1α stimulates the transcription of lactate dehydrogenase A (LDHA) and monocarboxylate transporter 4 (MCT4) in order to enhance the accumulated lactate excretion into the extracellular environment. Lactate and its related acidosis contribute to the indirect stabilization of HIF-1α and is able to preserve high ATP levels to resist cell death [52]. Moreover, due to the increased lactate transport, the Warburg effect is converted to oxidative phosphorylation in the lateral regions of GBM, while maintaining glycolysis in the inner core [62]. In addition to its contribution in lactate production and excretion, HIF-1α facilitates glutamine uptake and glutaminolysis as well, with lactate being the end product of the Krebs cycle. Interestingly, lactate acts as an antioxidant neutralizing the therapy-induced ROS thereby contributing to GBM resistance [63]. Treatment-induced ROS increase the stabilization of HIF-1α and upregulates PDK1, which limits the entry of pyruvate into the Krebs cycle and reduces the mitochondrial oxygen consumption. Furthermore, the increased ROS level activates the nuclear translocation of the nuclear factor erythroid 2–related factor 2 (Nrf-2), which binds to antioxidant response elements promoting the expression of antioxidant and anti-apoptotic genes such as heme oxygenase 1 (HO-1). Therefore, the hypoxia-induced metabolic shift to glycolysis protects the tumor from the increased ROS levels [64]. Consequently, this protective response may also induce mitochondrial autophagy mitigating the damages of ROS, thus enhancing resistance to conventional treatments [63]. Moreover, under hypoxic conditions, the transcription of genes such as peroxisome proliferator-activated receptor gamma (*PPARγ*) and fatty acid-binding proteins (*FABP*) 3 and 7 were upregulated by HIF-1α [65], provoking thus an increase in fatty acid synthesis and lipoprotein uptake. Furthermore, to avoid lipotoxicity induced by the accumulation of lipid molecules in the cytoplasm, HIF-1α stimulates the formation of lipid droplets [52]. Another regulator of aerobic glycolysis is p21, an inhibitor of cyclin-dependent kinases, which had been found to be upregulated under hypoxic conditions. A positive feedback loop was discovered involving p21 and HIF-1α, where the latter binds on the HRE of p21 to enhance its transcription and at the same time p21 promotes the transcription of HIF-1α to maintain its activity under hypoxia. The positive correlation between HIF-1α and p21 played an important role in upregulating GLUT1 and LDHA to mediate glycolysis as well as contributing to the radioresistant response of GBM [66]. On the other hand, protein arginine methyltransferase 3 (PRMT3) was found to be elevated in GBM, as it enhanced the expression of HIF-1α thus increasing glycolysis, and promoted cell cycle progression of GBM cell lines [67]. Therefore, the metabolic adaptation in GBM driven by hypoxia reduce the effect of oxidative stress on cellular death and reduces the ROS damaging capacity, leading to increased treatment resistance.

#### *Autophagy*

Upon tumor development and growth, more nutrients are required to compensate for the starvation and lack of oxygen occurring in these sites. Therefore, hypoxia is the main driver of autophagy, a highly conserved process among species. "Self-eating" or autophagy occurs mainly under a stressful environment or during starvation, which causes cellular degradation and the consequent formation of autophagosomes. Degradative organelles, such as lysosomes, engulf and process these double-membrane vesicles to initiate their breakdown and the recycling of their internal macromolecules. In cancer, the generated metabolite precursors, such as fatty acids, amino acids, and ATP, are used by tumor cells to perform essential survival processes [68, 69]. Hence, in late cancer stages, autophagy promotes tumor growth, due to its contribution as a recycling system for metabolite precursors where the resulting products provide extra nutrients for cancer cells [52]. In addition, autophagy has been described as a protective mechanism in GBM, as it prevents necrosis and contributes to chemo- and radioresistance [69]. In normoxia, several pathways interact to inhibit autophagy. For example, m-TOR is activated, while Bcl-2 or Bcl-$x_L$ couple Beclin1 to induce its inhibition. On the other hand, under hypoxia, HIF-1α plays a role in the induction of autophagy via the upregulation of *BNIP3* and *REDD1* gene transcription, which displaces Beclin1 from its inhibitory complex and activates m-TOR inhibitors, respectively [70]. Recently, studies have shown that yes-associated protein (*YAP*) overexpression increases transcription and translocation of HMGB1 from the nucleus to the cytoplasm and enhances autophagy in GBM driven by hypoxic conditions and starvation [71]. Interestingly, the in vitro knockdown of HIF-1α in GBM cell lines ameliorated their response to radiation, while silencing Beclin-1 reversed the hypoxia-induced radioresistance of these cell lines [72]. Moreover, kinases such as casein kinase 1α (CK1α) were found to increase the cellular viability and proliferation of GBM cell lines by binding to HIF-1α and positively regulating autophagy [73]. Furthermore, the epigenetic silencing of the tumor suppressor gene *ANKDD1A* through its hypermethylation has been linked to the downregulation of *FIH1* and consequently the transcriptional activation and stability of HIF-1α. In GBM, the CpG islands in the promoter region of *ANKDDA1* are frequently hypermethylated, yielding a low expression profile. Contrarily, the regain of *ANKDDA1* expression after demethylation treatment reduced the stability of HIF-1α through direct interaction with FIH1, inhibited autophagy mediated by hypoxia, and induced apoptosis in the hypoxic microenvironment [74].

#### *Immunosuppressive tumor microenvironment*

Several mechanisms are employed by GBM to evade the immune system, where most of the immune cells experience dysfunction despite the rare metastasis of GBM outside the brain. To start with, T cells undergo a range of dysfunctions such as senescence, tolerance, anergy, exhaustion, and ignorance all of which reduce the capacity of T cells to attack the solid tumor. As a result, the proliferative capacity of effector T cells is reduced through telomere shortening in senescence; T cells undergo apoptosis while regulatory T cells (Tregs) proliferate due to the tolerance mechanism. T cells express inhibitory immune checkpoints dependent on the exhaustion degree after repeated exposure under suboptimal conditions such as CTLA4, CD39, and PD-1. Namely, GBM cells express PD-L1 and can bind to PD-1 on T cells to inhibit their action. Interestingly, a positive correlation has been established between activated HIF-1α and the expression of PD-L1 [75]. T cells also experience anergy due to insufficient co-stimulation or continuous low-level antigen exposure, leading to decreased T cell activation. Finally, T cells that are prevented from antigen exposure by the BBB experience T cell ignorance [76]. Not only T cells are dysfunctional in GBM, but also other immune cells including natural killer and myeloid cells. The level of circulating myeloid-derived suppressor cells is very high in GBM and exerts an additional immunosuppressive effect on innate antitumor immunity. Likewise, cytokines secreted including TGF-β and Il-10 play a critical role in tumor progression and immunosuppression via the stimulation of Tregs [76]. Particularly, the interplay between GBM and its surrounding hypoxic microenvironment promotes the infiltration of some immune cells to increase tumorigenesis. For instance, the expression of CD276, a non-canonical immune checkpoint protein, in the neovascularization regions was found to be elevated. Notably, the immunoglobulin CD276 suppresses Th response and is usually absent in non-tumoral vessels [33]. These observations indicate that the immunosuppressive microenvironment in GBM is highly regulated by hypoxia. Moreover, the activation of HIF genes contributes to the increased secretion of VEGF and the upregulation of CXCL12, which in turn increases the tumor-associated macrophages (TAM-$CD11b^{high}CD45^{high}$) recruitment into solid tumors. In particular, HIF-2α is responsible of inducing an M2 immunosuppressive phenotype-like of polarized TAMs, leading as a result to immunosuppression, proliferation, angiogenesis, and local invasion [76]. Other ligand-receptor pairs that contribute to the polarization of TAMs to the M2-type were described in GBM. For instance, CSF1-CSF1R, ANXA1-FRP1 and 3, IL1B-IL1RAP, CCL3-CCR1, and CCL4-CCR1 have been reported to induce

M2-type TAM polarization in GBM [77]. In addition, studies confirmed that the upregulation of *CD162* gene, a downstream target in the CLOCK-HIF-1α-Legumain (LGMN) axis, enhances microglial migration and its infiltration into the tumor microenvironment and drives the immune-suppressive polarization of microglia as well [78]. Therefore, LGMN was considered a chemokine for microglia and a prognostic factor in GBM patients since its expression was increased in higher grades and was negatively correlated with GBM survival. Finally, inhibiting the CLOCK-OLFML3-HIF-1α-LGMN-CD162 axis increased the anti-tumor immunity and ameliorated the efficiency of the coupled anti-PD1 immunotherapy [78]. Furthermore, single-cell RNA-seq analysis revealed that hypoxia-induced *ALKBH5* transcription stabilizes a lncRNA NEAT1, through m6A demethylation, leading to the expression and secretion of CXCL8/IL8 to recruit the immunosuppressive TAMs in GBM [79]. In another study, single-cell mRNA sequencing of seven GBM patients was analyzed where most cells making up the tumor bulk were malignant cells and TAMs [77]. Moreover, six different cellular states have been identified including a subset with NPC2-like cellular state with proliferative and higher metabolic activities as compared to the other cellular states, indicating a possible origin in the developmental trajectory of GBM. Out of the six identified cellular states, two were correlated with the hypoxic niche, MES1- and MES2-like tumor cells. The MES-like state was shown to highly express several immune factors such as CXCL2, 3, 8, 12, and 14, IL-1B, IL6ST, and IL32 among others and were able to induce immune mediators including Il2/STAT5, TNFα, IFNα, and IFNβ response. This same subpopulation was also associated with high signaling levels of TGF-β and an elevated EMT activity even though they presented a quiescent and non-cycling state. Therefore, the MES-like state is mainly responsible of generating the immunosuppressive microenvironment surrounding GBM contributing to its poor prognosis [77]. Interestingly, hypoxia genes *HIF-1A* and *HIF-2A* were upregulated in both MES states, with the HIF-2α (EPAS1) being activated, indicating that MES-like tumor cells were under chronic hypoxia. Surprisingly, TAMs invading the tumor had a heterogeneous nature which was dependent on the different GBM subtypes. Thus, TAMs could be divided into 4 clusters based on their transcriptional profile and functions, where one of the identified clusters respond to low levels of oxygen in GBM. This TAM cluster (TAM-1) was also associated to a poor GBM prognosis since it has been highly correlated with the MES-like state and was found to be distributed in acute and chronic hypoxic niches as well as in the invasive niche. Another interesting discovery regarding the hypoxia-related effect on angiogenesis has been reached in this study. Only MES-like state GBM cells along with associated TAM-1 cluster expressed VEGFA revealing that hypoxia-dependent GBM cellular communication promote angiogenesis [77]. Moreover, GBM patients with lower MES-like and TAM-1 clusters signature score had a longer survival period, which displays the repercussion of hypoxia on the poor prognosis of GBM. It is noteworthy to mention that results from single cell RNA-seq analysis identified 3 co-expressed gene modules that were linked with an immunosuppressive subtype. The interaction of TAM, blood vessels, and tumor cells influenced the expressed modules leading to the development of a progressive GBM phenotype, with hypoxia being one of the key players in this interaction [80].

#### *Stem-like cell phenotype*

As described previously, GBM is most famous for its heterogeneous nature, not only within different patients or inter-patient but also within the tumor itself known as intratumoral heterogeneity. The presence of differentiated, quiescent, and GSCs within tumor bulk contributes to the increased resistance and recurrence rate of treated GBM. The expression of some stem cell markers that allow the identification of GSCs increased in the presence of hypoxia. These markers include CD9, CD133 (prominin-1), Olig2, integrin αβ, aldehyde dehydrogenase (ALDH), CD44, Sox2, Oct4, and nestin [57]. Interestingly, the single-cell RNA sequencing technique of patient-derived samples has helped decode the underlying aspects of GBM heterogeneity and its aggressive nature. For instance, results of scRNA-seq describes the presence of meta-signatures including hypoxia and cell cycle-related genes shared across different patients and even different cancer types [81]. Simultaneously, a set of unique and disease-specific signatures could be unraveled, and these represent a potential target for clinical therapies. When cultured under hypoxic conditions, patient-derived GBM cells exhibited a higher expression of stem cell markers such as *PROM1*, *FUT4*, and *SOX2* [82]. Furthermore, based on spatial transcriptomic analysis of GBM patient-derived samples, a spatial overlap was observed between the amplified chromosome 7 and hypoxia-related signature. Further analysis of these cells showed that their unique genetic dysregulation increased their migratory potential. Therefore, due to the remaining long-distance migratory cells after the surgical resection, an increased recurrence occurs. Noteworthy, based on scRNA-seq, the inner core of GBM represented more hypoxia- and adhesion-related genes driven by hypoxia and the subsequent overexpression of HIF-1α, in addition to the presence of a low proliferating population as compared to the tumor margins [81]. Also, under hypoxia, HIF-1α activates the JAK/STAT signaling

pathway and VEGF secretion, driving the self-renewal capacity of GSCs. Equally, TGF-β plays an essential role in promoting GSCs self-renewal, driving EMT, and tumorigenesis [57]. Moreover, under hypoxic conditions, the stem cell marker CD44 releases its intracellular domain that binds and stabilizes HIF-2α, to activate hypoxia target genes and induce stemness of GBM [83]. Interestingly, the silencing of HIF-2α was effective in reducing the stemness phenotypes of neurospheres formed in vitro and induced an additive effect with TMZ on GBM cellular viability [84]. On the other hand, there exist an interaction between the hypoxic microenvironment and its ECM on the maintenance of GSCs residing within this niche. As such, the upregulated HIF expression linked to hypoxic acidification maintains the stem-like profile of GSC, as well as the ECM components including laminin and tenascin-C (TN-C) among others, which facilitates progression [57]. Noteworthy, the interplay between HIF-1α and TGF-β increased the number of quiescent GBM organoids developed in vitro which exhibited self-renewal capacity and a higher resistance to RT and CT than differentiated GBM [85].

#### *Invasion and mesenchymal shift*

Invasion in GBM is associated with fast recurrence and a poorer OS. Hypoxia promotes invasion in GBM mainly through the activation of pathways implicated in ECM degradation and remodeling, EMT, and chemokines [41]. The interaction between tumor and surrounding cells governs the infiltrative capacity of GBM. Movement patterns, mainly single-cell movement, is the predominant infiltration mechanism of GBM. Under hypoxia, TGF-β regulates integrins αvβ3 and αvβ5 to promote tumor invasion through the activation of focal adhesion kinase (FAK) mediated by HIF-1α [41]. Similarly, the upregulation of MMPs under hypoxia and the downregulation of adhesion molecule NCAM or CD56 was linked to the increased invasiveness and migration of GBM [56]. Furthermore, remodeling the cytoskeleton is essential for creating a better migratory space away from the tumor for the cancerous cells that have acquired a reversible phenotypic change, i.e., EMT [86]. Signaling pathways such as Twist, Snail, Slug, and others participate in the induction of EMT. In addition, circulating myeloid cells release growth factors required for the EMT process in a hypoxic and acidic microenvironment [86]. Moreover, EMT interferes with the acquired radioresistance driven by TGF-β secretion and HIF-1α activation. Interestingly, an increased expression of stem cell markers by the activation of NOTCH and Wnt/β-catenin signaling pathways was observed in recurrent GBM emphasizing the role of hypoxia-induced EMT in the malignant progression of GBM [86]. In addition, the STAT3-mediated regulation of the migration factor ODZ1 or teneurin-1 (TENM1) increased the migration and invasion of GBM and was regulated by HIF-2α [87]. Finally, the activation of EGFR/PI3K/AKT pathway mediated by the transcription factor CCAAT enhancer binding protein delta (CEBPD) under hypoxia promoted GBM invasion and tumorigenesis [88]. Noteworthy, RNA-seq analysis revealed the contribution of *CEBPD*, *FOSL1*, *MXI1*, and *YY1* in the invasion of GBM under hypoxia [89].

Taking into consideration the mechanisms and pathways influenced by chronic hypoxia within the GBM and its microenvironment, it is evident why GBM exhibits a significant resistance capacity and recurrence potential. Therefore, in addition to the existing multimodal treatment options, such as radio- and chemotherapy, alternative therapeutic strategies aimed at targeting hypoxia may represent a potential clinical approach to counteract the adverse effects of hypoxia on the poor prognosis of GBM [90].

Table 1 summarizes some target genes of HIF-1α and HIF-2α in addition to the consequent biological contribution of hypoxia in GBM progression [4, 52, 57, 91].

### Proteomics-based analysis of hypoxia in GBM: towards a personalized treatment

The hypoxic microenvironment prevalent in GBM highly contributes to the alteration of several genes and their consequent protein expression. Therefore, the analysis of proteins and their interactions might serve as a predictive method of patient survival and a potential indicator of GBM pathogenesis. Given the critical role of hypoxia on the clinical outcome of GBM, several studies have employed a proteomics approach to study the effect of tumor hypoxia on protein modulation. For instance, a proteomics data analysis with 2348 quantifiable proteins showed that 62 proteins have been significantly altered, with 28 proteins being overexpressed and 34 underexpressed, in normal versus hypoxic GBM LN18 cell line [92]. The idea behind performing such analyses is to use the generated risk score that covers a range of preclinical and clinical aspects to help physicians choose the most suitable treatment for each patient. Based on proteomics analysis, 426 proteins presented alterations in the presence or absence of hypoxia. After converting these proteins into their corresponding genes, 212 annotated genes were found in GBM TCGA database. After performing a univariate Cox analysis followed by Lasso regression analysis, 7 genes were identified as hypoxia-related DEGs, including *FKBP2*, *GLO1*, *IGFBP5*, *NSUN5*, *RBMX*, *TAGLN2*, and *UBE2V2*. Accordingly, patients were divided into two categories, low and high risk, based on the prognostic risk model established. Consequently, results showed that low risk patients

**Table 1** Target genes and pathways of HIF-1α and HIF-2α and their consequent biological role in GBM

| Target genes of HIFs | Hypoxia-induced biological functions |
|---|---|
| VEGF-A, VEGF-B, VEGF-C, VEGF-D, PlGF, PDGF, ADM, ANGPTL4, ANGPT1 and-2, EPO, IGF2, Notch, DLL-4, NO, Ang-1 and-2 | Angiogenesis [4, 52, 57, 91] |
| GLUT1 and 3, LDH-A, MCT4, PFK1, HK2, TCN2, PDK1, ALDOA, ENO1-2, PGAM1, PFKFP, LAT1, BCAT1 | Metabolic shift to glycolysis and lactate production [4, 52, 57, 91] |
| FABP 3 and 7, CA9, CA12, PPARα, PPARγ | Fatty acid uptake and acidosis [52] |
| GOT1, Nox4 | ROS level reduction [4] |
| BNIP3, BNIP3L, DDIT4, REDD1, ANKDD1A, ATGA9 | Autophagy induction and cytoprotection [4, 52] |
| Snail, Slug, ZEB1 | E-cadherin downregulation and mesenchymal phenotype [52] |
| S100A10, uPAR, PAI-1, MMP-2, 9, 14, cathepsins, fibronectin, Keratins 14, 18, and 19 | ECM degradation and remodeling to facilitate migration and invasion [4, 52] |
| SOX10, CEBPD, MXI1 | Migration and invasion (mesenchymal shift)[57, 91] |
| PD-L1, CD47, CD39/CD73, NF-kB, STAT3 | Immune modulation [52] |
| CD133, TGF-β, PROM1, FUT4, SOX2, OCT4, Nestin, EZH2, Nanog, SHH, KLF4, cMYC, Olig2 | Stemness properties [4, 52, 57] |
| CCND1 | Cell cycle arrest [91] |

1st row: *VEGF* Vascular endothelial growth factor A, B, C, D, *PlGF* Placenta-like growth factor, *PDGF* Platelet-derived growth factor, *ADM* Adrenomedullin, *ANGPTL4* Angiopoietin-like 4, *ANGPT1 and 2*, angiopoietin 1 and 2; *EPO* Erythropoietin, *IGF2* Insulin-like growth factor, *DLL-4* Delta-like ligand 4, *NO* Nitric oxide, *Ang* Angiotensin-1 and 2

2nd row: *GLUT1 and 3* Glucose transporter 1 and 3, *LDH-A* Lactate dehydrogenase A, *MCT4* Monocarboxylate transporter 4, *PFK1* Phosphofructokinase 1, *HK2* Hexokinase 2, *TCN2* Transcobalamin 2, *PDK1* Pyruvate dehydrogenase kinase 1, *ALDOA* Aldolase A, fructose-bisphosphate, *ENO1-2* Enolase 1–2, *PGAM1* Phosphoglycerate mutase 1, *PFKFP* Phosphofructokinase 1 platelet type, *LAT1* L-type amino acid transporter, *BCAT1* Branched-chain amino acid transaminase 1

3rd row: *GOT1* Glutamic-oxaloacetic transaminase 1, *Nox4* NADPH oxidase subunit 4

4th row: *FABP3 and 7* Fatty acid-binding proteins 3 and 7, *CA9* Carbonic anhydrase 9, *CA12* Carbonic anhydrase 12, *PPARα and γ* Peroxisome proliferator-activated receptor α and γ

5th row: *BNIP3* BCL2 interacting protein 3, *BNIP3L* BCL2 interacting protein 3 like, *DDIT4* DNA damage inducible transcript 4, *REDD1* Regulated in DNA damage and development 1, *ANKDD1A* Ankyrin repeat and death domain containing 1A, *ATGA9* Autophagy-related 9 A

6th row: *ZEB1* Zinc finger E-box-binding homeobox 1

7th row: *S100A10* plasminogen receptor, *uPAR* receptor for the urokinase plasminogen activator, *PAI-1* Plasminogen activator inhibitor-1, *MMPs* Matrix metalloproteinases 2, 9, and 14

8th row: *SOX10* SRY-box transcription factor 10, *CEBPD* CCAAT enhancer binding protein delta, *MXI1* MAX interactor 1

9th row: *PD-L1* Programmed death-ligand 1, *NF-kB* Nuclear factor kappa B, *STAT3* Signal transducer and activator of transcription 3

10th row: *TGFβ* Transforming growth factor β, *PROM1* Prominin 1, *FUT4* Fucosyltransferase 4, *SOX2* SRY-box transcription factor 2, *OCT4* Octamer-binding transcription factor 4, *EZH2* Enhancer of zeste 2 polycomb repressive complex 2 subunit, *SHH* Sonic hedgehog, *KLF4* Krüppel-like factor 4, *cMYC* myelocytomatosis oncogene, *Olig2* Oligodendrocyte transcription factor 2

11th row: *CCND1*, cyclin D1

below 65 years, with *IDH*-mut GBM and who received a combination of chemo- and radiotherapy, had a better survival than other groups and hence a lower risk score. Moreover, proteomics-based analyses of hypoxia-induced alterations in tumor proteins may help identify novel tumor surface antigens as potential targets for GBM therapies, notably immunotherapy. For instance, hypoxia-modulated proteins such as CXADR, CD47, CD81, BSG, and FXYD6 have been identified as potential targets in GBM [93]. Interestingly, proteomic analysis on bevacizumab-resistant GBM revealed increased the secretion of collagen VI to facilitate invasion through integrins and β-catenin signaling. This mechanism was strongly associated with tumor hypoxic microvascular proliferation region where angiogenesis is increased as well [94]. Likewise, another proteomic analysis on U373 GBM cell lines demonstrated that hypoxia regulates the expression levels of the G protein coupled receptor 56 (GPCR56) and transglutaminase 2 (TG2), both of which participate in the mesenchymal transition of GBM [95]. Likewise, proteomic analysis aimed to identify hypoxia-modulated proteins in GBM have revealed that the transcription factor CEBPD is an essential key player in the regulation of hypoxia-activated pathways. CEBPD is activated by both HIF-1α and HIF-2α and is involved in GBM invasion by activating fibronectin 1 and the EGFR/PI3K pathway [88]. The initiation of a mesenchymal transition and the enhanced invasive capacity of GBM driven by hypoxia contribute to the resistance of GBM to conventional treatments and increases the recurrence rate. Finally, the proteomic analysis from secretions of the extracellular vesicles in U-87 MG cell line uncovered a set of nine proteins that were highly regulated by hypoxia and associated with the mesenchymal subtype of GBM. The hypoxia signature proteins include the insulin-like growth factor-binding protein 3 (IGFBP3), tissue factor

(F3), carbonic anhydrase 9 (CA9), solute carrier family 2 facilitated glucose transporter member 1 (SLC2A1), nucleolin (NCL), osteopontin (SPP1), monocarboxylate transporter 1 (SLC16A1), membrane-associated progesterone receptor component 1 (PGRMC1), and annexin A5 (ANXA5) [96].

The significance of exploring different signature patterns along GBM is of great importance for therapeutic decisions. To clarify, for the same anti-cancer agent used, GBM cells of different axes, i.e., MYC-enriched cells versus KRAS-enriched cells, will behave differently towards the treatment, where the first responds while the other resists, and vice-versa (e.g., parbendazole). In addition to the MYC and KRAS-axes, hypoxia was also implicated in inducing distinct responses of GBM to different inhibitors, where the action of such inhibitors was only driven under normoxia with little effect in hypoxic microenvironment (e.g., PDGFRB inhibitor) [33]. Finally, the identification of hypoxia-signature protein sets has significant clinical implications, as it can guide the pharmacological design of novel therapeutic molecules. By tailoring treatments based on individual signatures that reflect patient-specific inter- and intra-tumor heterogeneity, personalized trials targeting these identified protein signature sets might offer a new strategy to reduce the aggressiveness of GBM and to improve its response to first-line therapies.

## Conclusions

To date, GBM remains one of the most challenging tumors due to its location and aggressive behavior. The proposed treatment options have slightly ameliorated the PFS of GBM patients but not necessarily the OS such as but not limited to RT techniques (IMRT, VMAT…), anti-angiogenesis therapy, TTF, and immunotherapy. However, despite the severity of the first-line therapies and the addition of novel treatments, GBM still resist and recur in almost all cases. The implication of tumor hypoxia and its negative impact on GBM development and progression have been described on several occasions, yet hypoxia is still not considered neither in the clinical diagnosis of GBM nor in the management of recurrent cases. In this review, we described recent studies that have focused on hypoxia-induced activation of biological mechanisms in GBM to highlight the importance of targeting hypoxia in GBM both in the preclinical and clinical context. In addition, the identification of a hypoxia signature through transcriptomic and proteomic analyses might lead to the opportunity of personalizing the GBM treatment. Several options exist to target hypoxia namely, tumor reoxygenation, inhibition of cellular oxygen consumption, inhibition of hypoxia signaling pathways, and anti-angiogenesis therapy, in addition to indirect strategies including but not limited to carbon ion therapy and dose-escalation deposit in hypoxic regions. Finally, the clinical translation of hypoxia-targeted strategies is strongly encouraged due to the drastic effects of hypoxia on GBM outcome.

### Abbreviations

| | |
|---|---|
| GBM | Glioblastoma multiforme |
| TMZ | Temozolomide |
| RT | Radiotherapy |
| CT | Chemotherapy |
| TTF | Tumor-treating fields |
| PFS | Progression-free survival |
| OS | Overall survival |
| hRT | Hypofractionation radiotherapy |
| nRT | Normofractionation radiotherapy |
| MGMT | O6-Methylguanine DNA methyltransferase |
| IMRT | Intensity-modulated radiotherapy |
| VMAT | Volumetric modulated arc therapy |
| VEGF-A | Vascular endothelial growth factor A |
| TCGA | The Cancer Genome Atlas |
| IDH | Isocitrate dehydrogenase |
| TGF-β | Transforming growth factor β |
| GSCs | GBM stem-like cells |
| EGFR | Epidermal growth factor receptor |
| ECM | Extracellular matrix |
| MMPs | Matrix metalloproteinase |
| D-2-HG | D-2-hydroxyglutarate |
| HIFs | Hypoxia inducible factors |
| ADM | Adrenomedullin |
| MRI | Magnetic resonance imaging |
| BBB | Blood brain barrier |
| ROS | Reactive oxygen species |
| EMT | Epithelial-to-mesenchymal transition |

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### Authors' contributions

J.B.G composed, edited and finalized the manuscript. G.N. edited the manuscript. H.B edited and finalized the manuscript.

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### Data availability

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## Declarations

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