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Therapeutic potential of glioblastoma stem cells

Abstract

Primary and the secondary GBM, these are the two distinct disease entity. The genetic and the epigenetic background of these tumors are highly variable and the treatment procedure of these tumors is not curable because of the cellular heterogeneity and intrinsic ability of the tumor cells to invading healthy tissues. The fatal outcomes of these tumors promote the researchers to find out new markers associated with prognosis and treatment planning. Here we summarized the roles of glioblastoma stem cells in tumor progression and malignant behaviors of GBMs with attention to signaling pathways and molecular regulator involved in maintaining the glioblastoma stem cell phenotype. A better understanding of these stem-like cells is necessary for designing new effective treatments and developing novel molecular strategies to targeting glioblastoma stem cells treatment for these patients.

Keywords: stem cell, glioblastoma stem cell, therapy, hypoxia, mutation

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Abbreviations: GBM, glioblastomas; HSP-90, heat shock protein 90; Adv, adenovirus; HSV-1, herpes simplex virus-1; ESC, embryonic stem cell; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; MGMT, methyl guanine-dna methyl transferase

Introduction

Glioblastomas (GBM) the most common and lethal brain tumor with current standard therapies include tumor resection, adjuvant chemotherapy and chemoradiotherapy.[1,2] More than 150years ago, Virchow proposed an embryonic rest theory for the origin of cancer. In the 1920's Bailey and Cushing were proposed the relationship between gliomas and undifferentiated cells. Several groups demonstrated that brain tumors display a functional cellular heterogeneity with a potential hierarchy of differentiation.[1]

Stem cells have the capacity to self renewal, proliferate and differentiate into a variety of different cell types. Cancer stem cells have been identified in various tumor types, such as prostate tumors, pancreatic adenocarcinomas, colon carcinomas, hepatocellular carcinomas, melanoma, lung and breast cancers, osteosarcomas and brain tumors.[3]

Cancer stem cells, also known as tumor initiating cells or tumor propagating cells, are self renewing tumor cells that propagate tumors phenotypically similar to the parental tumor[1,4] and the cancer stem cell existence was suggested by Park et al. in 1971.[5] Cancer stem cells share some critical characteristic with normal stem cells, including the capacities for self-renewal, multi-lineage differentiation and maintained proliferation.[2] Ignatova et al. in 2002 discovered stem cell properties of human cortical glial tumors[5] and isolated precursor cells that capable of forming neurosphere *in vitro*. GBM express multipotent neural stem cell like cells and also contains neurons, astrocytes and oligodendrocytes within the tumor mass.[2] Cancer stem cells in malignant gliomas were called glioblastoma stem cells (GSCs). These cells have the potential to differentiate into astrocytes, oligodendrocytes and neurons. GBM stem cells may be resulted from genetic and epigenetic changes in neural stem/progenitor cells or the differentiated cells.[2] Glioblastoma cancer stem cells have; self renewal,[2] pluripotency, neurosphere formation,[3] proliferation, angiogenesis, invasion and modulation immune response,[2] marker expression, multilineage differentiation and high motility (Table 1).[6,7]

Table 1 Biological characteristics for normal, cancer stem cell and glioblastoma stem cells

	Normal stem cell	Cancer stem cell	Glioblastoma stem cell
Self Renewal	+	+	+
Differential Potential	+	+	+
Survival Ability	+	+	
Niche Specific Microenvironment Requirements	+	+	+
Specific Homing	+	+	
Multipotency	+	+	+
Angiogenesis			+
Invasion		+	+
Immune Response		+	+
Motility	+	+	+

Normal stem cells are physically located in specific physical and functional anatomical locations or niches that are essential for maintenance of self renewal and an undifferentiated state. Cancer stem cells in brain tumors reside in a perivascular niche that recapitulates a relationship between normal neural stem/progenitors and the vasculature.[1] Normal stem cells can assume a quiescent state that is regulated by the stem cell niche. Cells are not proliferating or stop after DNA damage has an enhanced chance of survival. Glioma stem cells express a variety of proteins that promote survival following cancer treatment, the major drug resistance protein, MGMT and anti-apoptotic genes such as FLIP, BCL-2, BCL-XL and cIAP1.[6]

The major problem of these malignancies is their highly infiltrative nature and extreme resistance to conventional treatments. Active tumor angiogenesis is one of the hallmarks of glioblastomas. Cancer stem cells promote the development of their own perivascular niche through the secretion of proangiogenic factors, prominently VEGF. Florid angiogenesis is a defining hallmark of glioblastomas but these tumors are also characterized by regions of pseudopalisading

necrosis which are hypoxic and the[1] florid neovascularization also plays a crucial role in providing nutrition and oxygen and removing waste to facilitate the rapid growth and progression of glioblastomas. The degree of vascularization is significantly correlated with tumor aggressiveness and clinical prognosis.[2]

Oxygen tension is tightly regulated in normal physiology and is an important signal in development with low oxygen tension associated with maintenance of undifferentiated cell state. Hypoxia promotes the self renewal of embryonic stem cells and prevents the differentiation of neural stem cells *in vitro*.[1] Hypoxia can promote the expansion of glioblastoma stem cells fraction and regulate expression of stem cell markers. Hypoxia may enhance tumor progression and therapeutic resistance through its promotion of a cancer stem cell phenotype and induction of VEGF and other pro-angiogenic factors.[2] Hypoxic and necrotic regions are common in solid tumors and their presence correlates with an aggressive clinical course. Previous studies assumed that the tumor necrosis was driven by hypoxia and this has been supported by the demonstration of striking up-regulation of HIF target genes in region immediately adjacent to the necrotic areas. Activation of HIF pathways by tumor hypoxia itself is the major cause of dysregulated tumor metabolism.[8] Cells preferentially utilized glucose carbon for palmitate synthesis under normoxic conditions; however, fatty acids produced under hypoxia were primarily synthesized from glutamine carbon via the reductive pathway and Metallo et al.[9] showed that the when IDH1 protein knockdown it mitigated the use of reductive glutamine metabolism for lipogenesis under hypoxia.[9] Solid tumors consist of heterogeneous cancer cells, as well as vasculatures, stromal elements and inflammatory cells. GBM displays intratumoral heterogeneity and cellular hierarchy not only morphologically but also in differentiation status.[2]

Different studies tried to generate a model of GBM and they hypothesized that the GBM tumor mass was a multilayer and the every tumor layer showed distinct characteristic. Pistollato et al.[10] describe GSCs distribution according to tissue hypoxic gradient, Piccirilla et al.[11] found a different behavior of GSCs derived from distinct tumor areas and Tafani et al.[12] demonstrated that the different pro-inflammatory gene expression of diverse tumor areas. The tumor cells are hypoxic, showed high activation of NF-kB and the expression of pro-inflammatory genes were high, the peri-tumor area showed the high activation of NF-kB and the pro-inflammatory genes expressions were low, the core region of the tumor was high proliferation capacity and clonogenic ability, the expression of differentiation markers were low and the genetic abnormalities not shared with the tumor periphery. The necrotic core of the tumor shows highly hypoxic conditions and this region of the tumor was highly enriched in GCSc and shows the expression of immature markers such as CD133 and Nestin, the *in vitro* studies showed that the necrotic core of the tumor resistant to the chemotherapy. The intermediate layer of the tumor was hypoxic enriched in GSCs, it showed the expression of mixed lineage markers and the *in vitro* studies showed that the region of the tumor was resistant to the chemotherapy. The periphery of the tumor was highly vascularized, GCSs were rare in this region, the differentiation markers were expressed and this region of the tumor was sensitive for chemotherapy and the proliferation index and the clonogenic ability were low. But in the normal brain there was no NF-kB activation and no pro-inflammation genes expression was showed.[13]

Specific cell surface markers in glioblastoma stem cells

Cell surface molecules are associated with the maintanence of glioblastoma stem cells and differentially expressed in glioblastoma Stem cells, such as CD133, CD15, A2B5, L1CAM,[2,6] nestin, Musashi-1, BMI1, SOX2, Id1 and Oct4.[14]

For the self-renewal, proliferation, survival and multi-lineage differentiation the stem cell transcription factors such as: Sox2, Oct4, Nanog, c-Myc, Olig2 and Bmi1 has a critical role. Bmi1 has been required for glioblastoma stem cell self-renewal.[14] Oct4, Sox2 and c-Myc contribute to the survival and self renewal of brain tumor stem cells.[1] Neural stem cells have been associated with repair after strokes, severe injuries and also suggested for the treatment of neurological disorders.[6]

CD133 was the first discovered cell surface marker for hematopoietic stem cells and is the one of the best-studied GSC markers to date. It is expressed in the both early postnatal brain and adult brain,[3] CD133 expression rapidly decreased during the cell differentiation and this characteristic could be used to identify and isolate stem cells formation.[3] A2B5 is a cell surface marker expressed on neural precursor cells in the adult human brain and on neural stem cells from subventricular zone of human embryos.[6]

L1CAM (L1, CD171) is a neuronal cell adhesion molecule and essential for the growth, migration during central nervous system development and survival of CD133 positive glioma stem cells.[15,16] Studies showed that the L1CAM regulates neural cell growth and survival. By using lentiviral-mediated short hairpin RNA interference in CD133 for targeting L1CAM, inhibits growth and neurosphere formation of GSCs. In glioblastoma stem cells L1CAM mediated signaling makes radioresistance. Although L1CAM is a therapeutic target for GBM therapy.[16]

Musashi is an RNA-binding protein[17,18] and has been correlated with the grade of the malignancy and proliferative activity in gliomas and melanomas.[6] Musashi proteins control the stem cell state through the translational regulation of target mRNAs and the Musashi family is a highly conserved RNA-binding protein group expressed in undifferentiated stem/precursor cells at both embryonic and adult stages.[3,17] Immune responses to GBMs can be potent because they secrete immunosuppressive factors such as TGF-β, VEGF, PGE2, B7-H1 and CCL2. Glioma stem cells disrupt tumor immuno-surveillance and result in both ineffective adaptive and innate immune responses. This is another mechanism that glioma stem cells help protect the tumor, which results in high rates of tumor recurrence and patient death.[6]

Therapeutic targets for glioblastoma multiforme

The target of the glioblastoma multiforme is the bulk of the tumor. Tumor recurrence is attributed to glioma stem cell therapy resistance, treatments that directly target glioma stem cells could yield long term cures. Hypothesize is that once the glioma stem cells have been eliminated, the bulk tumor would not be able to sustain itself and would disseminate. The most effective treatment procedures are the radiation and chemotherapy against the bulk tumor.[6]

Wnt family, Sonic hedgehog, Notch,[4] TGF-β, BMP signalling,[19] Homeobox family, B lymphoma Mo-MLV insertion region 1 homolog (Bmi-1), PTEN, telomerase, efflux transporters, EGF, micro-RNA and VEGF receptors are important for self renewal and differentiation of glioblastoma stem cell and they may be useful for targeted therapy in glioblastoma stem cells.[4]

TGF-β signaling is important for of self-renewal and the maintenance of perivascular glioblastoma stem cells, PI3K/Akt

signaling is promote self-renewal for glioblastoma stem cells *in vitro* and also important for proliferation and survival of GSCs, MAPK signaling important for the proliferation and survival of GSCs.[19] The Notch signaling pathway is an important regulator in normal development and adult stem cell maintenance and tumorogenesis in the brain. Inhibiting the Notch signaling pathway, Notch receptors, ligands and downstream pathways may target the glioma stem cell population. Because Notch signaling directly activates transcription of the stem cell markers in glioma, such as nestin.[6] Inhibitors of Notch pathway components represent promising therapeutic candidates in GBM.

Nestin is an intermediate filament protein produced in stem cells during development. Nestin has several important cellular functions, such as signaling, cytoskeleton organization and metabolism. Nestin is frequently expressed in glioblastoma and important in grading and clinical outcome.[3] Nestin+ and CD133+ brain tumor cells were located in the proximity of the tumors vascular system so glioma stem cells associated with a vascular stem cell niches.[6] Recent studies showed that the CD-133 positive GSCs resistant to the conventional anticancer therapies[3] and CD133 positivity have been postulated to be a glioma stem-cell marker. Failure to cure glioblastoma has been attributed to the fact that therapies are aimed at the tumor bulk without significantly harming tumor stem-like cells. CD133 positive brain cells could become therapy targets to eliminate brain tumors.

The Hedgehog pathway is a vital role for normal brain development, neural stem cell survival and also plays important role in glioma tumorigenesis,[6] cyclopamine treatment also inhibit the Hedgehog pathway and decreases the glioma stem cells.[20] The VEGF family and tyrosine kinase VEGF receptors are important in glioma angiogenesis and the treatment process may be targeting this vascular niche.[6]

Perivascular tumor cells and hypoxic conditions play a fundamental role in GBM growth and progression and the hypoxic microenvironment induce angiogenesis, cell migration and tumor resistance. This nature of the HIF represents a new molecular target to inhibit GBM malignant progression.

Current glioma therapies may fail to cure patients because of the glioma stem cell process mechanisms to evade treatments and enhance survival. The remaining cells which are escaping the therapy promote tumor re-growth.

HSP-90 (heat shock protein 90) is a molecular chaperone which plays an essential role in many cellular processes including cell cycle control, cell survival, hormone and other signaling pathways. It is important for the stress response and is a key player in maintaining cellular homeostasis.[21] HSP-90 inhibits the glioblastoma stem cells and synergize with radiation/TMZ, on the other hand the anti-epidermal growth factor receptors such as cetuximab, nimotuzumab are increase the radiosensitivity of the GSCs.[4] Kang et al. showed that the BCNU and chloride channel blocker combination promote apoptosis and sensitize gliomas to BCNU.[4]

The glioblastoma stem cells have potent angiogenic properties and can recruit vessels during tumourigenesis because angiogenesis is a critical factor for the development and growth of GBM. GBM stem cells secrete two potent angiogenic factors as VEGF and stromal Derived Factor-1 to promote angiogenesis and when anti-angiogenesis pathway was targeted the tumor cell proliferation, tumor size will be decreased.

Cellular based therapy for cancer is aimed to inhibit self renewing capacity of tumors and applications used agents to inactivate self renewing capacity. Viral delivery with replication restricted viruses and retrovirus, adenovirus (Adv) and herpes simplex virus-1 (HSV-1) are the more studied viral brain tumor therapy vectors,[22] delivery of suicide genes to convert prodrugs in the tumor and achieve tumor cell death, delivery of cytokine genes to activate and attract immune cells against the tumor, delivery of tumor-suppressor genes to reprogram tumor cells into apoptosis or delivery of conditionally-replicating viruses to specifically lyse tumor cells while sparing normal tissue.[23] Uzzaman et al.[22] showed that embryonic stem cell (ESC)-derived astrocytes conditionally expressing genes can be used to induce apoptosis in malignant glioma cells *in vitro*. The tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) gene has shown to induced apoptosis in a variety of tumor cells, including gliomas and they conclude that the TRAIL is a rare example because it kills cancer cells but not normal cells.[22] Brescia et al.[24] used lentivirus-mediated short hairpin RNA to silencing CD133 in human GBM neurospeares and they showed that the CD133 could be used as a therapeutic target in GBMs.

The neural stem cell usage for the oncolytic viruses carrier was first used by Herrlinger and colleagues. They used NSCs to carry conditionally-replicating HSV into pre-implanted cerebral gliomas and the possible advantages over the inoculation of viral particles: migratory NSCs may deliver the viruses at further distances within the tumor compared to virus alone; they can protect the viruses from the host immunosurveillance; and their own lysis removes them from the host after therapy.[25]

The immune system genes is the other widely tested transgene have been immune-boosting interleukins such as IL-4, IL-12 and IL-23 and NSC-mediated sustained delivery of interleukins was found more efficient than viral-based delivery *in vivo* and resulted in improved animal survival or the anti-angiogenic factors such as endostatin, thrombospondin-1 and the angiostatic factor PEX genes delivered by NSCs in glioma models.[23]

Glioblastoma stem cells and therapeutic resistance

GBM are highly infiltrative tumors and that display extreme resistance to conventional radiotherapy and chemotherapy. Studies showed that the glioblastoma stem cells contribute to the therapeutic resistance[2,13,26,27] because of the tumor heterogeneity, tumor contains different regions as mentioned above and sensitivity of the treatment of the each region is highly variable such as tumor periphery showed highly sensitive to the therapy but necrotic core or the intermediate core showed resistant to the treatment.[13] The region which surgical resection occurs or radiotherapy applied of the tumor is more important because of the heterogeneous structure of the glioblastomas.

GSCs represent important therapeutic targets because they have intrinsic machinery that overcomes current chemoradiotherapeutic approaches. Some of the molecular mechanisms underlying GSC resistance to chemoradiotherapy are discussed below. The GSCs discovery may help to the explanation of aggressiveness, relapse and treatment resistance of glioblastoma. The DNA repair capacity and the resistance to radiation induced apoptosis of the glioblastoma stem cells more than the non stem tumor cells and glioblastoma stem cells were rapidly recover the genotoxic stress.[2] This means the treatment procedure which makes genotoxic stress is not proper the treatment choice of these tumors.

Recent studies showed that the low molecular weight inhibitor of Chk2 and Chk1 kinases repeals the radioresistance of glioblastoma

stem cells and suggesting that the targeting the DNA damage checkpoint activation may sensitize these stem cells to radiotherapy.[2]

Multiple molecular mechanisms regulate the glioblastoma stem cells radioresistance. Recent studies suggested that targeting Sir T1 expression or HSP90 activity abate radioresistance of glioblastoma stem cells. Inhibition of the Notch signaling pathway by the γ-secretase inhibitor or Notch shRNA makes glioblastoma stem cells more sensitive to radiation and Notch pathway is the another potential target for reducing GBM radio-resistance.[2] And recent studies showed that the GSCs can initiates gliomagenesis via activation of NOTCH signaling pathway,[3] if NOTCH pathway silenced or knockdown the genes in this pathway may help to prevent the tumor recurrence.

According to the aggressive nature of glioblastoma the active tumor angiogenesis is also one of the hallmarks of these tumors. GBM stem cells promote therapeutic resistance and metastasis. GBM stem cells stimulate tumor angiogenesis by expressing elevated levels of VEGF and contribute to tumor growth, which has been translated into a useful therapeutic strategy in the treatment of recurrent or progressive GBMs. GBMs rarely metastasize beyond the central nervous system, these highly infiltrative cancers often invade into normal brain tissues preventing surgical resection and GBM stem cells display an aggressive phenotype. Targeting GBM stem cells may effectively reduce tumor recurrence and significantly improve GBM treatment.[2]

Two groups of alkylating agents are commonly used for treatment glioblastoma in the clinic: TMZ and nitrosoureas. O6-methylguanine-DNAmethyltransferase (MGMT) is an important DNA repair enzyme that contributes to glioblastoma resistance to temozolomide. The epigenetically mediated silencing of the MGMT gene in GBM has been shown to correlate with an increased survival. Moreover, a correlation with outcome independently of treatment choice, i.e. chemotherapy or radiotherapy.[28] Recent studies showed that the MGMT status has no predictive values in primary glioblastomas.[29] Eramo et al.[30] were the first to investigate the chemoresistance of GBM CSC than Beier et al.[31] described that TMZ may selectively deplete clonogenic and tumorigenic cells in a dose-dependent manner whereas it hardly affected overall viability and they conclude that the cells with stem cell-like properties were selectively depleted irrespective of the CD133 or MGMT status. Liu et al.[32] showed that when the cells are CD133+ they significantly less decreased than the viability of CD133- tumor cells when treated with TMZ and the cells with MGMT methylated shows CD133 and it was a stem cell-like properties and also Pistolatto et al.[10] showed higher resistance of central, hypoxic CD133 CSC as compared to cells derived from the periphery due to increased MGMT expression. Bralten et al.[33] showed that there was no CD133 expression detected in secondary GBMs which are derived from lower grade gliomas and suggesting that the IDH1 mutations mostly found in secondary GBMs and might inhibit the growth of GBM cells *in vitro*.

MGMT methylation highly occurs in secondary GBMs and used to be a predictive marker for TMZ treatment in the light of these data we assume that the GSCs which high CD133 expressions and resistant to TMZ are the primary GBMs which are directly derived from neural stem cells and the tumors which shows lower CD133 expression and treated successfully with TMZ are the secondary GBMs and it derives from lower grade astrosytomas. On the other hand restricted oxygen conditions increase the CSC fraction and promote acquisition of a stem-like state and multiple HIF-regulated genes are preferentially expressed in glioma stem cells in comparison to non-stem tumor cells and normal neural progenitors. When we look at the previous studies, Lai et al.[34] showed that the glioblastomas with IDH mutation may originate from lineage committed neural cells and the gliomas without IDH mutation may arise from neural stem cells. As we know IDH1 mutation occurs highly in secondary GBM which derived lower level astrocytomas, This means IDH1 mutant, CD133- and MGMT + cells are secondary GBMs and CD133+ and IDH1 wild type tumors are primary GBMs. If the IDH1 is mutant on these tumors and it causes high hypoxia and hypoxia induced GSCs proliferation and this makes the resistant to the therapy. Several studies have demonstrated that GBM cells that are CD133 negative are still capable of tumor initiation and some GBM tumors do not contain any CD133-positive cells[35–37] the cause of this situation is the precursor cell type because of the primary and secondary GBMs are developed different progenitor cells and effected different genetic pathways (Figure 1).

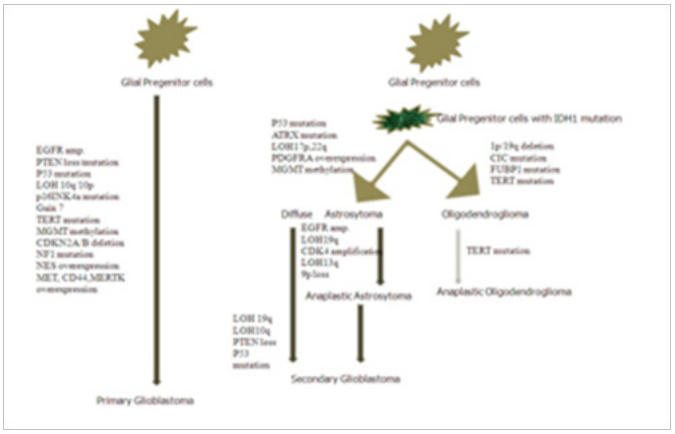

Figure 1 A cartoon demonstrating the pathways of primary and secondary GBMs.

Hypoxia is common in many types of solid tumors. Hypoxic conditions have a negative impact on tumor growth. Hypoxia also enhanced the tumor progression and therapeutic resistance and promotes tumor angiogenesis and cancer invasion. The HIF1α and HIF2α controlled the VEGF expression in glioblastoma stem cells. Hypoxia can promote the expansion of glioblastoma stem cells and also prevents the differentiation of neural stem cells and promotes the maintenance of self-renewal potential of embryonic stem cells.[2] Heddleston et al.[38] showed that the restricted oxygen conditions increase expression of glioblastoma stem cells and glucose is used as a primary carbon source for mammalian tissues but Metallo and colleagues used knockdown IDH1 and show that the when the cells under hypoxia the knockdown of IDH1 protein mitigated the use of reductive glutamine metabolism for lipogenesis under hypoxia[9] so cells survive and proliferate under hypoxic conditions. Hypoxia is associated with the tumor growth, progression and resistance to conventional therapy of glioblastoma and also known to support the survival of non-neoplastic neural stem cells and glioblastoma stem cells (GSCs) which are the second greater drug resistance, selfrenewal potential and tumorigenicity *in vivo*.[39]

Cancer stem cells in brain tumors reside in a perivascular niche that recapitulates a relationship between normal neural stem/progenitors and the vasculature[1] and this means GSCs are maintained within a hypoxic niche; hypoxia plays a key role in the initiation, progression and recurrence of GBM (Figure 2). On the other hand Notch pathway is essential for hypoxia-mediated maintenance of GSCs, either depletion of HIF-1a or inactivation of Notch signaling

partly inhibited the hypoxia-mediated maintenance of GSCs. Li et al.[40] showed that hypoxic environment increased the expression of CD133 and nestin which are markers of CSCs, but reduced the proportion of cells positive for GFAP, a marker for differentiation of stem cells and Li an colleagues concluded that hypoxia could de-differentiate the differentiated glioma cells and promote the acquisition of stemness in these cells. But these studies are *in vitro* and *in vivo* studies are required to show the specific mechanism for hypoxia and treatment resistance and future studies will be aimed to identify the target for the treatment of glioma.

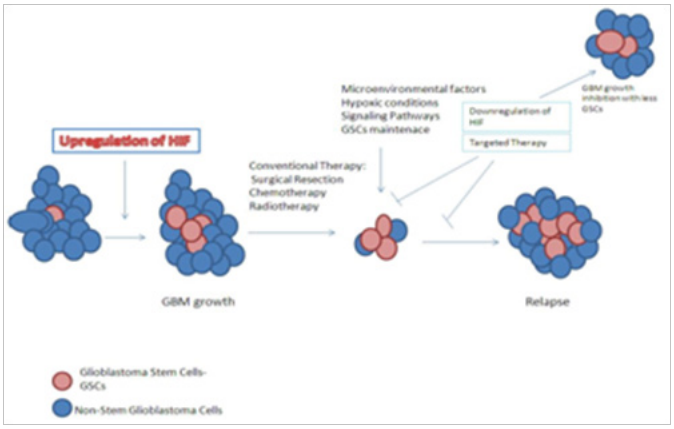

Figure 2 Hypothesized effects of conventional therapies that target progenitor cells that will result in recurrence vs. targeting glioblastoma stem cells which may be slower in debulking the tumor but will have a longer curative effect.

Conclusion

Glioblastoma is the one of the deadliest forms of cancer. The discovery of the major genetic alterations in glioma progress has made a major contribution to the understanding of the molecular pathways involved in gliomagenesis. Current studies focus on defining specific markers that will facilitate their identification and isolation in tumor and using these markers for a therapy target. In this review we discuss the glioblastoma stem cell phenotype, characteristics and markers that used to identify glioblastoma stem cells and the genetic basis of resistance to treatment. In the light of these observations we outline strategies for the successful eradication of GSCs, including targeting the cellular pathways and cell surface markers. Finally, we summarized the therapeutic importance of these cells. Due to their high tumorigenic potential and resistance to current therapies, GSCs represent critical drug targets and we discuss the hypoxia, GBM progression, initiation, recurrence and maintained the phenotype of glioma stem cells (GSCs) under hypoxic conditions. Future studies will be focused on defining the HIFs mediated GSCs survival and treatment procedures and HIFs might be taken as the promising molecular target approaches for GBM therapeutics.

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Conflict of interest

The author declares no conflict of interest.

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