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Glioblastoma stem cells: a therapeutic challenge

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Abstract: The outcome for glioblastoma patients remains extremely poor, despite the advances in surgical and medical fields. It is hypothesized that glioblastoma progression, as well as tumor recurrence, is driven by a small number of cells called cancer stem cells (CSCs), which are characterized by their ability of self-renewal and proliferation, giving rise to progeny of transformation into multiple neuroepithelial lineages. Understanding the biology of CSCs is likely to explain why existing treatment strategies fail to affect the relatively quiescent and resistant CSC compartment. Here, we review the current knowledge on CSCs in glial tumors. In addition, we discuss the importance of the CSC hypothesis in the advancement of therapies for brain tumors.

Key words: Glioblastoma, cancer stem cell, therapy, resistance

1. Introduction

Glioblastoma is considered as the most aggressive primary brain tumor and has an extremely poor prognosis. The median 5-year survival rate is less than 3%, which makes this disease a devastating condition for both patients and their caregivers. Resistance to available therapies and recurrence are common in most cases; identification and molecular characterization of cancer stem cells (CSCs) (Singh et al., 2003; Yuan et al., 2004) have shown that these cells are responsible, in part, for resistance, as well as tumor reformation.

In this review, we will try to summarize the recent advances in glioblastoma biology, with a special focus on glioblastoma CSCs (GSCs). We will also discuss the molecular features of GSCs, and how these features can be exploited as potential therapeutic strategies.

2. Glioblastoma

2.1. Background and epidemiology

Gliomas are the most common primary brain tumors (approximately 80% of all cases). Glioblastoma in particular is the most common and aggressive form of glioma (Omuro and DeAngelis, 2013). Glioblastoma is classified as WHO grade IV astrocytoma according to the World Health Organization (WHO) classification of brain tumors. The estimated incidence of brain and nervous system tumors is 240,000 cases per annum. According to the Central Brain Tumor Registry of the United States (CBTRUS) report in 2013, the incidence of glioblastoma is 3.19/100,000. and the median age at diagnosis is 64 years (Ostrom et al., 2013; Thakkar et al., 2014).

The tumor is generally localized in the forebrain (cerebrum). In most cases, tumor formation occurs spontaneously. However, several genetic and epidemiological risk factors have been identified, including increased age, exposure to high-dose radiation, and history of genetic disorders (e.g., Li–Fraumeni syndrome, Turcot's syndrome, retinoblastoma, and neurofibromatosis 1 and 2), allergies, ionizing radiation, and occupational exposure to chemicals (e.g., pesticides, solvents) (Schwartzbaum et al., 2006; Ostrom et al., 2014). In addition, while symptoms vary between patients depending on tumor size and localization, the common symptoms include increased intracranial pressure, visual impairment, seizures, sensory loss, mood/personality changes, and impaired cognitive function (Wen and Kesari, 2008; Ostrom et al., 2014). Magnetic resonance imaging (MRI) is performed in suspected cases, and definitive diagnosis is made after pathological examination of the biopsy specimen.

Glioblastoma patients have poor prognosis, and the 5-year survival rate after diagnosis is less than 5% (Ostrom et al., 2013; Smoll et al., 2013). Long-term survival in

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glioblastoma is associated with several parameters, including younger age, lower Ki-67, hypermethylation of the O-6-methylguanine-DNA methyltransferase (*MGMT*) promoter, and mutations in the *IDH1* and *IDH2* genes (Scott et al., 1999; Krex et al., 2007; Yan et al., 2009; Hartmann et al., 2010).

2.2. Molecular subtypes of glioblastoma

In 2010, Verhaak and colleagues integrated mRNA expression profiles from different platforms and discovered four distinct molecular subtypes (Verhaak et al., 2010). They demonstrated that the so-called classical, mesenchymal, proneural, and neural subtypes are characterized by individual genetic signatures. The classical subtype is characterized by chromosome 7 amplification (especially *EGFR* gene amplification) paired with chromosome 10 loss, lack of *TP53* mutations, focal 9p21.3 deletion (on the *CDKN2A* locus), and high expression levels of *NES*, Notch, and Sonic hedgehog (SHH) signaling pathway elements (Verhaak et al., 2010). The mesenchymal subtype is characterized by focal deletion of 17q11.2 (*NF1* locus), expression of previously described mesenchymal markers (*CHI3L1* and *MET*), and high levels of TNF and NF-κB pathway elements (Verhaak et al., 2010). The proneural subtype is characterized by mutations in the *IDH1* and *PDGFRA* genes, which are not commonly seen in other subtypes (Verhaak et al., 2010). Integrated pathway analysis of genomic alterations in glioblastoma has shown that the PI(3)K/MAPK pathway, p53 pathway, and Rb pathway are the most affected signaling pathways (Brennan et al., 2013).

2.3. Standard of care

The current standard of care consists of gross total resection (GTR), followed by radiotherapy and adjuvant chemotherapy. GTR is the recommended approach as it reduces intracranial pressure (Ricard et al., 2012), and the extent of GTR has a significant effect on survival. Orringer and colleagues reported that 1-year survival is significantly higher in patients who undergo >90% resection (Orringer et al., 2012).

Temozolomide (TMZ) is the current standard for chemotherapy for glioblastoma. Stupp and colleagues showed that TMZ administration during and after radiotherapy significantly increases the median overall survival and 2–5-year survival compared to radiotherapy only (Stupp et al., 2009). Moreover, inactivation of *MGMT* expression through promoter methylation is correlated with higher sensitivity to TMZ (Esteller et al., 2000; Hegi et al., 2005). Hegi and colleagues identified that TMZ+radiotherapy leads to significantly higher median overall survival in patients with methylated MGMT promoter (21.7 months), compared to patients who received radiotherapy only (15.3 months) (Hegi et al., 2005). Taken together, these findings indicate the significant benefit offered by TMZ treatment to glioblastoma patients with a methylated MGMT promoter.

External beam radiation therapy (EBRT) is the current standard in radiotherapy. Resistance to radiotherapy is quite common, and it is known that a specific EGFR variant (EGFRvIII) mediates radioresistance by inducing the genes involved in double-stranded DNA repair mechanisms (Mukherjee et al., 2009).

3. Glioblastoma stem cells

Different models have been proposed to explain the complicated nature of tumor development. The CSC hypothesis postulates a hierarchy in the tumor population, where CSCs are positioned at the top. Thus, CSCs give rise to different cell types through differentiation (Tang, 2012). However, it should be noted that the relationship between CSCs and differentiated tumor cells is bidirectional; in vitro and in vivo interventions (treatment modalities, silencing/overexpressing genes and/or proteins, hypoxia) may trigger dedifferentiation of tumor cells to GSCs, thus creating a dynamic equilibrium between these cell populations (Tang, 2012).

3.1. Molecular features of GSCs

Long-term clonal repopulation and self-renewal represent two key features of CSCs (Nguyen et al., 2012). CSCs cannot be easily distinguished from differentiated tumor cells in the case of tumors that display low levels of hierarchy, and in relatively homogeneous tumors (Nguyen et al., 2012; Kreso et al., 2014).

GSCs share several features with neural stem cells (NSCs), including expression of Nestin and CD133, and can form spheres in the presence of required growth factors (Zhu et al., 2014). Similar to NSCs, GSCs also rely on certain transcription factors, which are crucial for their maintenance. These factors include sex-determining region Y-box 2 (SOX2), octamer-binding transcription factor (OCT4), and Nanog homeobox (NANOG) (Schmitz et al., 2007; Ikushima et al., 2011). A list of key biological markers that are used for characterization of GSCs is provided in the Table.

CD133 has served as one of the most frequently used markers to characterize GSCs. $CD133^+$ glioblastoma cells are able to form tumors, even in low cell numbers. Singh and colleagues reported that 100 $CD133^+$ cells are able to form tumors when transplanted into the brains of severe combined immunodeficient (SCID) mice; on the other hand, injection of high numbers of $CD133^-$ cells (10^5) does not cause tumor formation (Singh et al., 2004).

Contrary to this notion, different studies have shown the presence of $CD133^-$ GSCs (Beier et al., 2007, 2011). Comprehensive gene expression studies on molecular subtypes of glioblastoma have shown that CD133 positivity is enhanced in the mesenchymal subtype (Phillips et al., 2006).

Table . Important markers for characterization of GSCs.

Marker	Function	Reference
CD133	Positive association with aggressiveness	Brescia et al. (2013)
CD44	Positive association with aggressiveness	Pietras et al. (2014)
CD15	Enrichment marker in $CD133^-$ tumors	Kahlert et al. (2012), Auffinger et al. (2014)
TLX	Self-renewal	Zou et al. (2012)
ID1	Self-renewal	Soroceanu et al. (2013)
Integrin α6	Regulation of self-renewal, proliferation, and tumor formation	Lathia et al. (2010)
L1CAM	Maintenance of growth and survival of $CD133^+$ cells	Bao et al. (2008)
Nestin	Regulation of sphere formation, tumor growth, invasion	Matsuda et al. (2015)
SOX2	Maintenance of self-renewal	Seymour et al. (2015)
Osteopontin	Maintenance of stemness, sphere formation, tumor growth	Lamour et al. (2015)

SOX2 is a transcription factor that has a critical role in maintenance of self-renewal of stem cells, and especially neural stem cells (Ellis et al., 2004; Thiel, 2013). SOX2 overexpression at mRNA and protein levels has been identified in tumor tissues (Alonso et al., 2011; Annovazzi et al., 2011). Gene amplification (Brennan et al., 2013), as well as hypomethylation of the SOX2 promoter (Alonso et al., 2011), can explain SOX2 overexpression in glioblastoma. GSCs express SOX2, which maintains stemness through the TGF-β signaling pathway (Ikushima et al., 2009). Gangemi and colleagues showed that loss of SOX2 expression impairs cell proliferation and tumorigenicity of glioblastoma cells in vivo (Gangemi et al., 2009). Given the limited expression of SOX2 in the adult brain (Baer et al., 2007; Seymour et al., 2015), targeting SOX2 can be a potential strategy for treatment of glioblastoma.

Previously, Chen and colleagues showed that ablation of Nestin-expressing glioblastoma cells leads to increased survival of tumor-bearing mice (Chen et al., 2012). This finding highlights the importance of Nestin with respect to tumor propagation.

TLX is a nuclear receptor that is specifically expressed in adult NSCs, and its presence is required for neurogenesis in the subventricular zone (SVZ) (Liu et al., 2008). In addition, mouse models of glioblastoma have shown that the combination of forced TLX overexpression and loss of tumor suppressor genes (TP53 and INK4A/ARF) is sufficient to cause tumor formation (Liu et al., 2010; Park et al., 2010; Zou et al., 2012). Liu and colleagues identified that TLX overexpression leads to migration of progenitor and/or stem cells from their natural niche, and combination of p53 mutations and TLX overexpression lead to glioblastoma initiation in vivo (Liu et al., 2010). While Tlx has been shown to be druggable (Benod et al., 2014), identification and characterization of potent Tlx inhibitors warrant further studies. However, given the close link between TLX and histone deacetylases (HDACs), HDAC inhibitors can be used to target TLX^+ GSCs (Xie et al., 2014).

L1CAM is a neural adhesion molecule that regulates different cellular processes, including migration, invasion, adhesion, survival, and growth (Maness and Schachner, 2007). Bao and colleagues showed that L1CAM is differentially overexpressed in $CD133^+$ glioblastoma cells (Bao et al., 2008). Cheng and colleagues provided supporting evidence for this phenomenon, showing that L1CAM is differentially overexpressed in the invasive fronts of glioblastoma (Cheng et al., 2011). In another study, Held-Feindt and colleagues showed that TGF- β1 signaling regulates L1CAM expression in glioblastoma, and L1CAM confers resistance to TMZ (Held-Feindt et al., 2012). L1CAM also participates in regulation of DNA damage checkpoint response. Cheng and colleagues showed that the intracellular domain of L1CAM is cleaved from the membrane-bound form through ADAM10- (A Disintegrin and Metalloprotease 10) and Presinilin-mediated cleavage. This, in turn, leads to its translocation to the nucleus, where it induces NBS1 expression through c-Myc (Cheng et al., 2011). As a result, L1CAM enhances DNA damage checkpoint activation and confers radioresistance to GSCs.

Osteopontin is a secreted phosphoprotein that is critical for osteoblast function (Jan et al., 2010). In addition to its role in bone formation, osteopontin is an important angiogenic molecule for glioblastoma, as it is found in the tumor microvasculature (Takano et al., 2000). Osteopontin also functions as a driver of

invasion and tumor growth. Jan and colleagues showed that osteopontin enhances invasion of glioblastoma cells by inducing MMP-2 secretion and vimentin expression (Jan et al., 2010). They also demonstrated that 5-aza-2'-deoxycytidine, an anticancer agent, reduces cell invasion and inhibits glioblastoma tumor growth by suppressing osteopontin expression (Jan et al., 2010).

Inhibitor of DNA binding/differentiation (Id) proteins are negative regulators of the basic HLH family of transcription factors (Perk et al., 2005). Id proteins are well known for their functions related to differentiation, as well as self-renewal of stem cells (O'Brien et al., 2012; Romero-Lanman et al., 2012; Lasorella et al., 2014). Id1 overexpression is a common feature of different cancers (Perk et al., 2006; Lasorella et al., 2014) and is also related to metastasis of breast cancer to the lungs (Gupta et al., 2007). Id1 overexpression has been documented in glioblastoma, which is also positively correlated with tumor grade and proliferation index (Vandeputte et al., 2002). Given the link between TGF-β and Id1 expression, inhibition of TGF-β signaling can be a potential strategy for glioblastoma treatment. Indeed, Anido and colleagues found that inhibition of the TGF-β signaling pathway decreases the number of $CD44^{high}/Id1^{high}$ GSCs through suppression of Id1 and Id3 expression (Anido et al., 2010). They concluded that this strategy can be employed to overcome tumor recurrence, which is driven through GSCs.

3.2. Deregulated signaling pathways in GSCs

Deregulation of cellular signaling pathways is one of the major features that distinguishes CSCs from NSCs. Previous studies have shown that several key signaling pathways are deregulated in glioblastoma. These include Notch signaling, Wnt/beta-catenin signaling, receptor tyrosine kinase (RTK) signaling. and Sonic hedgehog (SHH) signaling.

RTK signaling pathways have been extensively studied in glioblastoma. Of note, comprehensive genetic analyses have shown that EGFR gene amplification and activating mutations, as well as PDGFR amplification, are common events in glioblastoma (Verhaak et al., 2010). Activation of RTK signaling pathways leads to constitutive activation of the downstream PI3K/Akt signaling, which is responsible for maintenance of cell growth and proliferation.

Notch signaling is critical for stem cells, as it functions in regulation of self-renewal and differentiation. Tchorz and colleagues showed that constitutive activation of Notch signaling leads to tumor formation and astroglial lineage entry (Tchorz et al., 2012).

SHH signaling is a key pathway for maintenance of self-renewal and regulates proliferation of GSCs (Clement et al., 2007; Xu et al., 2008; Takezaki et al., 2011). In addition, hyperactivation of SHH signaling and PTEN coexpression are associated with reduced survival (Xu et al., 2008). Bar and colleagues showed that cyclopamine-mediated inhibition of SHH signaling depletes GSCs (Bar et al., 2007). Their findings suggest that SHH inhibition can be a potential strategy to specifically target GSCs.

CSCs also rely on Wnt/beta-catenin signaling to regulate stemness and differentiation. In addition, Kim and colleagues reported that activation of Wnt/beta-catenin signaling can contribute to radioresistance in GSCs (Kim et al., 2012). Thus, targeting Wnt/beta-catenin signaling may serve as an alternative therapeutic strategy. Recently, De Robertis and colleagues identified and characterized a small molecule inhibitor (SEN461) of the canonical Wnt/beta-catenin signaling pathway. They found that in vivo administration of SEN461 reduces tumor growth (De Robertis et al., 2013).

4. Glioblastoma stem cells: a therapeutic challenge

It is hypothesized that treatment failure results from insufficient drug delivery and the targeting of differentiated tumor cells rather than CSCs (Beier et al., 2011). CSCs use different mechanisms to escape chemotherapy-induced cell death, including activation of DNA damage response (Bao et al., 2006) and functions of specific proteins including MGMT and multidrug resistance proteins (e.g., ABCB1) (Beier et al., 2011).

Another factor affecting chemoresistance is tumor evolution. It has been suggested that tumors adapt a chemoresistant phenotype through selection of preexisting clones or formation of de novo subclones (Prados et al., 2015). Supporting evidence for this notion has come from a recent study, where Johnson and colleagues analyzed the origin and evolution of recurrent glioma (Johnson et al., 2014). Through exome sequencing, they identified that TMZ treatment causes a significant portion of the recurrent tumors to follow an alternative path to high-grade glioma. Moreover, they found that recurrent tumors have a TMZ-induced mutagenesis signature (in RB and Akt-mTOR genes) (Johnson et al., 2014).

In another study, Auffinger and colleagues demonstrated for the first time that glioblastoma cells are capable of interconverting between non-CSCs and CSCs upon chemotherapy (Auffinger et al., 2014). They showed that TMZ treatment increases the proportion of GSCs in vitro and in vivo. In addition, lineage-tracing analysis showed that this increase is not a result of enhanced cell proliferation, but rather a result of a phenotypic shift to the CSC state (as demonstrated by stem cell markers, including CD133, SOX2, Oct4, and Nestin). Overall, their results suggest a potential mechanism for escape from chemotherapy.

Targeting self-renewal capacity of GSCs has been used as a promising strategy for treatment of glioblastoma. Recently, Hale and colleagues showed that GSCs are

enriched in CD36 (a scavenger receptor), which can be used to distinguish self-renewing cells. In addition, they found that reduction of CD36 expression leads to loss of self-renewal, tumor initiation capacity, and loss of integrin alpha 6 expression. Overall, they concluded that glioblastoma CSCs selectively use CD36 for their maintenance (Hale et al., 2014).

Deregulated miRNA expression (i.e. downregulation of tumor-suppressor miRNAs) can also contribute to tumor formation and/or progression in glioblastoma. Gal and colleagues compared miRNA expression profiles of $CD133^+$ and $CD133^-$ glioblastoma CDCs and found that several miRNAs (including miR-451, miR-486, and miR-425) are overexpressed in $CD133^-$ glioblastoma CDCs compared to $CD133^+$ cells. They also showed that exogenous overexpression of miR-451 disperses neurosphere formation and inhibits cell proliferation (Gal et al., 2008). Their results indicate that restoring expression of tumor-suppressive miRNAs can be used as an alternative strategy for treatment of glioblastoma.

Hitomi and colleagues reported that connexins, which are structural elements of gap junctions, show differential expression between GSCs and differentiated tumor cells (Hitomi et al., 2015). Their results show that differentiated tumor cells predominantly express connexin 43 (Cx43), whereas GSCs express Cx46. In addition, they found that Cx46 expression decreases and Cx43 expression increases during differentiation of GSCs. Reduced expression of Cx46 impaired the tumor-forming capacity and self-renewal of glioblastoma CSCs.

Gamma-secretase inhibitors and RNA interference have been previously used to inhibit Notch signaling, which leads to reduced radioresistance and impaired formation of tumor spheres (Fan et al., 2010; Wang et al., 2010).

5. Concluding remarks

The advances in stem cell biology in the past decade have helped us to better understand the development and pathogenesis of brain tumors. Despite the identification and characterization of GSCs, the existence of a specific cell population and their specific roles in the context of tumor development are still debated. By utilizing the key molecular features of CSCs, new therapeutic strategies can be developed to achieve more durable clinical responses. Alternatively, given the inherent tumor tropism of NSCs, endogenous and/or engineered NSCs can be used as therapeutic delivery vehicles for treatment of glioblastoma. Taken together, translating the accumulated knowledge on the biology of NSCs and CSCs with well-designed studies may bring new possibilities for effective therapies to glioblastoma patients.

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