Glioblastoma Multiforme Stem Cell Characteristics

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Abstract

Glioblastoma Multiforme (GBM) is the most common and most aggressive malignant primary brain tumour in humans. There is a poor prognosis associated with GBM tumours which is due to therapeutic resistance and tumour recurrence after surgical removal. GBM are comprised of a heterogeneous population of cells and it has become clear that a subpopulation of stem cells are responsible for tumour initiation and maintenance. There is a wide variety of GBM stem cells markers, although as yet, there is no single marker that can be used to identify all stem cells with complete specificity and sensitivity. A number of signalling pathways and genetic mutations have also been distinguished in GBM stem cells, although their use in translational therapeutics remains in their infancy.

Introduction

Glioblastoma Multiforme (GBM)/Glioma Grade IV is the commonest type of primary brain tumour in adults and is also the least successfully treated solid tumour. GBM can develop de novo (primary GBM) or from a pre-existing lower grade glioma (secondary GBM). Although Glioblastomas are relatively rare, with an incidence of 2–3 cases per 100,000 people in Europe

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and North America, the median survival time of patients following surgical resection, radiation and temozolomide (TMZ) therapy remains stubbornly low at 14 months (Johnson and O'Neill 2012). Compared with the advances in treatment of other solid tumour types, the prognosis for GBM patients has improved minimally over the past decades, thus underscoring the challenges and complexities in early detection and effective treatment.

Cellular Markers

Early evidence for the existence of GBM stem cells came from isolating the cell fraction expressing the neural stem cell surface marker CD133 (Singh et al. 2003). The CD133+ cells could differentiate in culture into tumour cells that phenotypically resembled the tumour from the patient from which the cells originated. The CD133+ stem cell was the first marker of its type for GBM and continues to be the primary cell marker used in research. Over time there have been a variety of other cell markers investigated, many of them cell surface markers (L1CAM, SSEA-1, intregrin α-6, A2B5, CXCR4) but also intracellular markers (nestin, sox-2) have also been used to locate and target the GBM stem cells with varying degrees of success.

CD133 is a pentaspan transmembrane glycoprotein cell surface molecule, whose expression on cancer stem cells can be found in a variety of human malignancies including renal, prostate, colon, hepatocellular, and pancreatic as well as neural tumours including GBM (Mizrak et al. 2008). The cell marker is located in the membranous protrusions of the plasma membrane and whose role, although not fully understood, is speculated as being a regulator of the plasma membrane structure, specifically maintaining an appropriate lipid composition within the plasma membrane (Mizrak et al. 2008). Evidence illustrates that CD133+ GBM stem cells are capable of multi-lineage differentiation, can form neurospheres and imitate tumours which closely resembles the patient's tumour, as well as express neural stem cell genes nestin, Msi-1, CXCR4 and maternal embryonic leucine zipper kinase.

CD133 remains the most popular cell marker for GBM stem cell location and isolation. Despite this, the sensitivity of this marker remains unclear. Research has shown that not all cells in GBM specimens are CD133+, nor does every GBM specimen contain CD133+ cells. In CD133+ GBM tissue samples, the percentage of all cells which are CD133+ is varied. In one study, analysis between GBM samples showed that between 2 and 60 % of the total cells were CD133+ (Ogden et al. 2008). However, such variability between samples was noticed in the earliest studies by Singh and colleagues who showed that immunestainting of GBM xenografts revealed islands of CD133+ cells amid large groups of CD133− cells; indicating that not every cell in the xenograft is CD133+ (Singh et al. 2004). Furthermore, when a small number of purified CD133+ cells were transplanted, a heterogeneous primary xenograft grew, which consisted of a minority of CD133+ with the remaining cells being CD133−. Therefore showing that CD133+ cells can generate CD133− cells and thus suggesting a cell hierarchy exists within a tumour (Singh et al. 2004).

Evidence therefore shows that CD133 does not have to be present nor is it likely to be present in every cell of a GBM for the marker to still remain highly specific as a stem cell marker. However, in an analysis of 16 primary GBM obtained directly from patients, three of the specimens did not contain any detectable levels of CD133+ cells (Ogden et al. 2008). Other studies have shown greater consistency of CD133+ cells being present in GBM tumours, including an analysis of 47 patients with GBM of which 45 contained at least 1 % of CD133+ cells (Zeppernick et al. 2008). It is likely then, that CD133 is not present in every GBM and therefore its use a stem cell marker in this particular type of tumour is not absolute.

The overall relevance of CD133 is not yet understood, and there is growing evidence that the marker may have significant limitations as well, including that CD133+ cells are found in normal brain tissue (Zhang et al. 2008). A reliable

cell marker has the potential to be used as a target for therapeutic purposes, which could not be the case for CD133 if significant amounts are found outside of tumour tissue. Furthermore, the exact association of CD133 and GBM stem cells remains a concern. A study looked at GBM tumour samples from 20 patients who were divided into two groups, CD133+ high group (CD133+ cell ratio >3 %) and a CD133+ low group (CD133+ ratio <3 %), whereby the patients' clinical characteristics were analysed using MRI scan data (Joo et al. 2008). It was found that as compared to CD133-high GBMs, CD133-low GBMs had a tendency to be localised within the deeper structures of the brain and show more invasive growth patterns and ventricle involvement and also the rate of disease progression after chemotherapy and radiotherapy was relatively higher in the CD133-low GBMs compared to the CD133-high GBMs. Indicating that the relationship between CD133 as a marker and GBM tumours is not straight forward.

A cellular marker which has an intimate relationship with CD133 is L1CAM. This marker is a transmembrane protein, specifically a neuronal cell adhesion molecule and a member of the L1 protein family. It is known to be involved in axon guidance and cell migration. In addition, abnormal expression of this protein has been found in several different human cancer types including colon, melanoma, breast, ovarian, renal and neuroblastomas. Analysis of three human glioma surgical specimens through fluorescence-activated cell sorting (FACS) indicated a strong correlation between CD133+ cells and L1CAM+ cells, in that where one marker was present, so was the other (Bao et al. 2008). In contrast the vast majority of CD133− cells were L1CAM negative (>99 %). L1CAM could therefore be as useful a marker as CD133.

Furthermore, when L1CAM is targeted using lentivirus expressing shRNA, there is a 90 % reduction in its expression, and a significant decreased in the ability of CD133+ GBM cells to form neurospheres occurs, with no effect on CD133− GBM cells and their ability to form neurospheres (Bao et al. 2008). Interestingly, L1CAM over expression in a number of solid tumours has been linked to cancer invasion. When primary GBMs have been examined for L1CAM expression using immunohistochemical staining, it has been found that L1CAM expression is enriched in the population of cancer cells in the invasive fronts of tumours (Cheng et al. 2011). Although a subpopulation of cancer cells inside tumours also expressed L1CAM, the invasive fronts showed many more cells with high levels of L1CAM. This suggests that preferential expression of L1CAM in GBM stem cells may be closely associated with the elevated invasive potential of stem cells in GBM tumours. This indicates that cell markers may not only discern stem cells from non-stem cells but also specific characteristics/roles of that stem cell.

The antigen Stage-Specific Embryonic Antigen-1 (SSEA-1/CD15/LeX) is a fructose-containing trisaccharide adhesion molecule that can be expressed on glycoproteins, glycolipids and proteoglycans. SSEA-1 has been used in the recognition of certain leucocyte malignancies including Hodgkin's disease and histiocytosis. Research into its use as a potential GBM stem cell marker is extremely limited but remains promising. Analysis of GBM tumour cell lines has found SSEA-1 expression in 23 of the 24 evaluated tumours, with the percentage of expression ranging from 0.7 to 87.5 % (Son et al. 2009). When the SSEA-1 cells were grown they formed a population of cells which contained the properties to meet tumour stem cell criteria (formed neurospheres, were clonogenic in agar, were highly tumourgenic in vivo) and secondary tumours could be produced when SSEA-1+ cells were extracted from the SSEA-1+ cell-generated orthotopic tumours with the same characteristics as the primary tumour.

Integrin α6 is another cellular marker that although has not been researched extensively in the GBM stem cells evidence as a potential marker is intriguing. Integrin α6 is the receptor for the extracellular matrix protein laminin, and in the brain both of these molecules regulate neural stem cell growth, as well as playing a pivotal role in maintaining adhesion to the ventricular zone, ensuring proper neural stem cell division (Lathia et al. 2010). Previous evidence has shown

that laminin-coated flasks in neural stem cell culture media has been used to isolate and expand GBM stem cells successfully (Pollard et al. 2009) which indicated a possible role for the laminin-integrin relationship in GBM development. In an assessment of GBM surgical biopsy specimens labelled with antibodies against integrin α6, 60 % of the integrin-positive GBM cells were located within 5 μm of a blood vessel (Lathia et al. 2010) indicating invasive properties, especially in the peri-vascular areas. Furthermore, functional assays to define GBM stem cells, which included self-renewal assays, expression of stem cell markers, and tumour propagation, showed that the stem cells expressed significantly higher levels of intregrin α6 compared to matched non-stem cell glioblastoma cells (Lathia et al. 2010).

Difficulties arise in being able to narrow down the most useful GBM stem cell markers because it is uncommon for cell markers to be directly compared to one another. Interestingly, when A2B5 (a cell surface ganglioside that marks a fraction of human subcortical white matter cells that have neural stem cell properties) was compared CD133 in 16 GBM specimens the percentage of cells containing the marker was found to be higher (between 33–90 % vs. 2–60 %) and unlike CD133, A2B5+ cells were found in every specimen (Ogden et al. 2008). When the GBM specimens where FAC-sorted by cell marker (A2B5+CD133−, A2B5+CD133+, A2B5−CD133−) 92 % of the A2B5+CD133− grafts formed xenografts once injected into rats, indicating a clear subset of GBM cells with tumourigenic properties. It is unfortunate that A2B5-CD133+ were so rare that they could not be retrieved by FACS in this study. Despite this the results suggest that A2B5 has promise as an additional or even alternative GBM stem cell marker.

An interesting receptor which has been shown to be over expressed in primary Glioblastoma progenitor cells and is also associated with the recruitment of GBM stem cells is CXCR-4. This receptor is a seven transmembrane G-coupled protein alpha-chemokine receptor specific for stromal-derived-factor-1 (SDF1) and plays an important role in lymphocyte trafficking. CXCR-4 receptors have not only been found to be over expressed in GBM progenitor cells but also its protein ligand, CXCL-12, promotes a proliferative response in these cells (Ehtesham et al. 2009). Further evidence indicates that through SDF-1-CXCR-4 signalling GBM stem cells are recruited to perivascular niches and are induced to become pericytes, predominantly by transforming growth factor β (Cheng et al. 2011). CXCR-4 and SDF1 are also specifically expressed on neo-vessel endothelial cells within the tumour and levels of both increase with increasing grade of astrocytoma (Rempel et al. 2000). The location of SDF1 has led to the hypothesis that the interaction of SDF1 in the GBM tumour and CXCR-4 which is also present on both macrophages and CD8+ T cells, may explain why the immune system fails to mount a successful immune response. SDF1 binds to the CXCR-4 receptor on both macrophages and CD8+ T cells, in which both cells secrete TNF and TNF-R respectively; macrophages and T cells bind to each other through TNF/TNF-R interaction which induces a death signal in the T cell, thereby limiting an immune response to the tumour (Rempel et al. 2000).

Intracellular molecules have also been used as GBM stem cell markers with two of the more successful ones being Nestin and Sex determining region Y Box-2 (Sox-2), although, for obvious reasons the study of live cells is more difficult for intracellular markers when compared to their extracellular counterparts. Sox-2 is a member of the Sry-related High Mobility Group Box family of transcription factors. Like many of the other GBM stem cell markers it is not specific for grade IV gliomas and has been found in all WHO glioma grades (Knights et al. 2012). Interestingly, studies have shown that silencing of Sox-2 expression by RNA interference lead to the loss of tumourigenicity and proliferation of GBM stem cell lines and also it has been found that arsenic trioxide inhibits self-renewal and induces apoptosis in GBM stem cells through the down-regulation of SOX-2 (Knights et al. 2012). Nestin, an intermediate filament protein involved in the organisation of the cytoskeleton, appears to be a more reliable intracellular marker than SOX-2. Nestin has been shown to be positively correlated with higher grade gliomas and higher expression

levels have also been predictive of significantly lower 5-year survival rates (Knights et al. 2012).

Signalling Pathways

A commonly activated signalling cascade in many human malignancies including GBM, is the Akt pathway. Akt, also known as Protein Kinase B (PKB), is a serine/threonine-specific protein kinase which regulates numerous tumour-associated processes, including cell growth, cell cycle progression, survival, migration, and angiogenesis. Upstream regulators of this pathway include epidermal growth factor, PTEN deletion and PIK3CA mutations. Evidence continues to grow regarding the influence of this signalling pathway on GBM development, with early evidence already showing that the pathway is activated in the majority of GBM; its activation has been associated with reduced patient survival times; and that A-443654 (an Akt inhibitor) when delivered locally either at the time of tumour implantation or in a delayed fashion prolonged survival in an experimental intracranial rodent Glioblastoma Multiforme model (Gallia et al. 2009). Importantly, A-443654 also inhibits GBM stem-like cells with similar efficacy compared with traditionally cultured GBM cell lines indicating no observable stem cell specific resistance (Gallia et al. 2009). Other molecules which target the Akt pathway include KP-372-1 and KP-372-2, which been shown to inhibit the *in vitro* growth of six GBM cell lines, where the reduced activation of Akt downstream targets including GSK-3β and p70s6k, caused a decrease in cell growth which stemmed from the induction of apoptosis (Koul et al. 2006).

Notch signalling pathway is a highly conserved signalling network, which is critical for a series of processes in stem cells, including cell fate specification, differentiation, proliferation, and survival. The deregulation of this pathway is found in many cancers including, acute lymphoblastic leukaemia, ovarian and colorectal cancer as well as GBM. Notch signalling is initiated when transmembrane ligands on one cell bind Notch receptors on an adjacent cell and cause the γ-secretase-mediated proteolytic release of the Notch intracellular domain (NICD). NICD then translocates into the nucleus where it interacts with the transcriptional cofactor CBF1 and activates targets such as HES and HEY genes, which modulate neuronal and glial differentiation (Fan et al. 2010).

In Notch pathway blockade through γ-secretase inhibitor (GSI-18) tumour growth was slowed in GBM neurosphere cultures and it was additionally found that Notch pathway blockade with GSI-18 also reduced the percentage of cells expressing the stem/progenitor cell markers CD133 and Nestin in GBM neurospheres and that these cells were no longer able to efficiently form colonies in *vitro* or engraft in *vitro*, consistent with the concept that a key subpopulation of cells required for efficient tumour propagation was no longer present (Fan et al. 2010). Retinoic acid (RA) also shows potential in affecting GBM stem cell growth following its successful use in the treatment of acute promyelocytic leukaemia. Retinoic acid treatment on GBM stem cell neurospheres resulted in rapidly induced morphological changes, induced growth arrest, decreased cyclin D1 expression and increased p27 expression, as well as reduced stem cell markers including CD133, Msi-1, nestin and Sox-2, and overall decreased neurospheres-forming capacity (Ying et al. 2011). There is further evidence to suggest that RA can also impair the secretion of angiogenic cytokines and disrupt GBM stem cell motility (Campos et al. 2010).

Recent studies point to a role for the Hedgehog signalling pathway in regulating adult stem cells involved in maintenance and regeneration of adult tissues. Sonic Hedgehog (SHH) signalling specifically plays a key role in regulating vertebrate organogenesis including the growth of digits on limbs and the organisation of the brain. In adults the SHH-Gli1 pathway controls cell division of stem cells and when it is activated or maintained inappropriately, various tumours can develop, including those in the skin, muscle and brain (Ruiz et al. 2002). Early results have shown that SHH-Gli pathway has a general role in controlling progenitor cell number in the developing dorsal brain (Dahmane et al. 2001).

Furthermore, it has been demonstrated that deregulation of SHH-Gli signalling in the CNS

leads to hyper-proliferation of the precursor cells and suggests its involvement in the initiation and maintenance of brain tumourigenesis (Dahmane et al. 2001). This raises the possibility that tumours are derived from such cells which have abnormal SHH-Gli signalling, possibly even stem cells, which are unable to differentiate and/or stop proliferating (Ruiz et al. 2002). More recently, it has been demonstrated that GBM and their cancer stem cells require SHH-Gli pathway activity for proliferation, survival, self-renewal, and tumourigenicity (Clement et al. 2007). It has also been found that cyclopamine, an antagonist of Gli1, can induce cell death in GBM cell lines, although after its removal culture recovery occurred, indicating a temporary cytostatic and cytotoxic effect (Clement et al. 2007), moreover cyclopamine in combination with TMZ has shown a synergistic effect in GBM stem cell cytotoxicity.

Signal transducer and activator of transcription 3 (STAT3) regulates diverse cellular processes, including cell growth, differentiation and apoptosis and is frequently activated during tumourigenesis. Constitutive activation of STAT3 has been observed in many human cancers including; breast, head and neck, prostate, melanoma and thyroid cancer (Sherry et al. 2009). Inhibition of STAT3 in GBM stem cells irreversibly abrogates neurosphere formation and inhibits proliferation and also causes the down-regulation of genes associated with the neural stem cell phenotype, providing evidence that STAT3 regulates multipotency in these cells (Sherry et al. 2009). GBM stem cells treated with STAT3 inhibitors (SAT-21 and S31-201) have shown a reduction in stem cell markers olig2 and nestin, and have failed to proliferate, although the cells did not undergo apoptosis, nor is there evidence that STAT3 inhibition alone is sufficient to induce complete differentiation of GBM stem cells (Sherry et al. 2009).

Genetics

The Cancer Genome Atlas data reports that *p53* as one of the most commonly mutated tumour suppressor genes. Lower estimates suggest that *p53* mutations are found approximately 30 % of the time in all grades of gliomas (Li et al. 2009) although higher estimates argue that *p53* is nearly invariably altered in sporadic gliomas through either point mutations that prevent DNA binding or loss of chromosome 17p (Furnari et al. 2007). The importance of *p53* in gliomagenesis is also underscored by the increased incidence of gliomas in Li-Fraumeni syndrome, a familial cancer-predisposition syndrome associated with germline *p53* mutations (Furnari et al. 2007). Furthermore, it has also been found that concomitant central nervous system specific deletion of *p53* in the mouse generates a penetrant acute-onset high grade malignant glioma phenotype with notable clinical, pathological and molecular resemblance to primary GBM in humans (Zheng et al. 2008). It has also been found that through inhibiting *p53* function via RNAi in stem cells derived from GBM specimens, these stem cells became significantly more sensitive to TMZ (Blough et al. 2011). Although the molecular basis for the differing effects of *p53* status on sensitivity to TMZ is as yet unknown.

The *Bmi-1* gene was originally identified as a collaborating oncogene in c-Myc induced lymphomagenesis, and is a member of the Polycomb group (PcG) gene family of chromatin modifiers and transcriptional repressors (Bruggeman et al. 2005). When *Bmi-1* is over-expressed in adult forebrain, NSCs expand dramatically and continue to proliferate aggressively. It has been shown that the polycomb factor *Bmi-1* represses cell-cycle inhibitors *p16*, *p19*, and *p21* which are necessary for NSC self-renewal (Cui et al. 2010). Also it has been argued that the up regulating of *Bmi-1* coordinates the down regulation of miRNA-128 which is also found to inversely correlate with WHO tumour grade (Cui et al. 2010).

The phosphatase and tensin homolog (*Pten*) gene mutation is a step in the development of many cancers. *Pten* acts as a tumour suppressor gene through the action of its phosphatase protein product which is involved in the regulation of the cell cycle, particularly in preventing cells from dividing and growing too rapidly. In analysis of 35 clinically annotated human primary GBM samples 40 % had *Pten* missense mutations,

insertions, deletions or splicing mutations (Zheng et al. 2008). *Pten* mutation results in uncontrolled PI3K signalling. More recently, integrated transcriptomic profiling, in *silico* promoter analysis and functional studies of murine neural stem cells established that dual, but not singular, inactivation of *p53* and *Pten* promotes an undifferentiated state with high renewal potential and drives increased Myc protein levels and its associated signature (Zheng et al. 2008).

Amplifcation of the *EGFR* gene and the subsequent over-expression of EGFR protein is one of the most common genetic alteration in GBM, with a frequency of about 40 %, with the most common of the EGFR mutations is the EGFRvIII (Gan et al. 2009). The mutation results in the loss of exons 2 to 7 of the *EGFR* gene, resulting in an in-frame deletion of 267 amino acids in the extracellular domain (Gan et al. 2009). EGFRvIII appears to enhance tumourigenicity through multiple mechanisms including; enhancing cell proliferation by promoting P13K/Akt signalling, Shc and Grb2 association and Ras activity; inhibiting cell cycle regulators such as $p27^{KIP1}$; promoting survival in cells by increasing expression of anti-apoptotic proteins such as Bcl-X_L; and enhancing angiogenesis and cell invasion by unregulating vascular endothelial growth factor, interleukin-8 and matrix metalloproteinase 13 expression (Gan et al. 2009).

More recently, High-mobility group A1 (*HMGA1*) transcriptional and translational expression in GBM stem cells (determined by the presence of NSC markers CD133 and nestin) was found to be significantly higher than in GBM non-stem cells (determined by the presence of glia fibrillary acidic protein but not CD133 or nestin) (Fan et al. 2011) implying that its expression may be correlated with malignant proliferation, invasion and differentiation. The underlying mechanisms that *HMGA1* plays in tumourgenesis is not yet known. The gene codes for a non-histone chromatin protein which takes part in regulation of inducible gene transcription, DNA replication, heterochromation organisation, integration of retroviruses into chromosomes and metastatic progression of cancer cells.

There is growing evidence that GBM can be sub-classified based on their gene expression signature and that expression profile predict better outcome than histological class (Phillips et al. 2006). In one report GBM tissue samples could be separated into three molecular subtypes (proneural, proliferative and mesenchymal), through the analysis of 35 signature genes. The proneural subtype is distinguished by markedly better prognosis and expressed genes associated with normal brain tissue and processes of neurogenesis. Two poor prognosis subtypes, characterised by a resemblance to either highly proliferative cell lines or tissues of mesenchymal origin, show activation of gene expression programs indicative of cell proliferation or angiogenesis, respectively (Phillips et al. 2006). In an analysis of nine different GBM cultures established under neural stem cell conditions, two major phenotypes could be established (Gunther et al. 2008). One group of four cell lines were characterised by the expression of neurodevelopmental genes, showed a multipotent differentiation profile along neuronal, astroglial and oligodendroglial lineages, grew spherically *in vitro*, expressed CD133 and formed highly invasive tumours *in vivo* (Gunther et al. 2008). The other group of five cell lines shared expression signatures enriched for extracellular matrix-related genes, had a more restricted differentiation capacity, contained fewer or no CD133+ cells, grew semi-adherent or adherent *in vitro* and displayed reduced tumourigenicity and invasion *in vivo* (Gunther et al. 2008).

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