Glial Progenitor-Like Phenotype in Low-Grade Glioma and Enhanced CD133-Expression and Neuronal Lineage Differentiation Potential in High-Grade Glioma

Johan Rebetz¹, Dongping Tian¹, Annette Persson⁵, Bengt Widegren¹,³, Leif G. Salford¹, Elisabet Englund⁵, David Gisselsson⁴, Xiaolong Fan¹,²*

¹The Rausing Laboratory, Division of Neurosurgery, Lund University Hospital, Lund, Sweden, ²Strategic Research Center for Stem Cell Biology and Cell Therapy, Lund University, Lund, Sweden, ³Department of Cell and Molecular Biology, Lund University, Lund, Sweden, ⁴Department of Clinical Genetics, Lund University Hospital, Lund, Sweden, ⁵Department of Pathology, Lund University Hospital, Lund, Sweden

Abstract

Background: While neurosphere- as well as xenograft tumor-initiating cells have been identified in gliomas, the resemblance between glioma cells and neural stem/progenitor cells as well as the prognostic value of stem/progenitor cell marker expression in glioma are poorly clarified.

Methodology/Principal Findings: Viable glioma cells were characterized for surface marker expression along the glial genesis hierarchy. Six low-grade and 17 high-grade glioma specimens were flow-cytometrically analyzed for markers characteristics of stem cells (CD133); glial progenitors (PDGFRα, A2B5, O4, and CD44); and late oligodendrocyte progenitors (O1). In parallel, the expression of glial fibrillary acidic protein (GFAP), synaptophysin and neuron-specific enolase (NSE) was immunohistochemically analyzed in fixed tissue specimens. Irrespective of the grade and morphological diagnosis of gliomas, glioma cells concomitantly expressed PDGFRα, A2B5, O4, CD44 and GFAP. In contrast, O1 was weakly expressed in all low-grade and the majority of high-grade glioma specimens analyzed. Co-expression of neuronal markers was observed in all high-grade, but not low-grade, glioma specimens analyzed. The rare CD133 expressing cells in low-grade glioma specimens typically co-expressed vessel endothelial marker CD31. In contrast, distinct CD133 expression profiles in up to 90% of CD45-negative glioma cells were observed in 12 of the 17 high-grade glioma specimens and the majority of these CD133 expressing cells were CD31 negative. The CD133 expression correlates inversely with length of patient survival. Surprisingly, cytogenetic analysis showed that gliomas contained normal and abnormal cell karyotypes with hitherto indistinguishable phenotype.

Conclusions/Significance: This study constitutes an important step towards clarification of lineage commitment and differentiation blockage of glioma cells. Our data suggest that glioma cells may resemble expansion of glial lineage progenitor cells with compromised differentiation capacity downstream of A2B5 and O4 expression. The concurrent expression of neuronal markers demonstrates that high-grade glioma cells are endowed with multi-lineage differentiation potential in vivo. Importantly, enhanced CD133 expression marks a poor prognosis in gliomas.

Introduction

Gliomas, the most common primary tumors in the adult central nervous system, are currently classified according to their morphological features, into low- and high-grade glioma. Cells of low-grade (I and II) gliomas are well differentiated with clear histological similarity to astrocyte or oligodendrocyte lineage. High-grade (III and IV) gliomas are more anaplastic, with features resembling immature astrocytes, oligodendrocytes or a mixture of both types. Low-grade gliomas are frequently diagnosed in relatively young patients and many of these eventually develop into anaplastic gliomas which subsequently progress to the so-called secondary glioblastoma (GBM). In contrast, the GBM in older patients are mostly diagnosed as de novo without any clinically detectable history. It is unclear whether the early stages of de novo GBM development resemble low-grade gliomas. Although previous studies suggested glioma expression of oligodendrocyte progenitor cell antigen NG2 and PDGFRα, and transcription factor Olig1/2 [1–3], the lineage commitment and the stage of differentiation blockage of glioma cells are not clarified [4].

For decades, the median survival of high-grade gliomas has not been significantly improved [4]. In efforts to identify crucial
As demonstrated in previous studies, although gene expression of cancer cells reflects genetic and epigenetic alterations, a considerable fraction of this gene expression can nevertheless be characteristic of the non-transformed cell-of-origin. This principle has been applied to distinguish between leukemias originating from either stem cells or from progenitor cells by characterization of cancer initiating capacity and cancer-characteristic genetic mutations in these cell fractions [31,32]. In this study, we characterized freshly isolated low-grade and high-grade glioma cells for expression of surface markers characteristic of the glial lineage differentiation hierarchy and analyzed the expression of these markers in relation to patient survival. We also studied the glioma cell multi-lineage differentiation potential in vivo by assessing GFAP and NSE/synaptophysin expression in the corresponding formaldehyde fixed specimens.

Materials and Methods

Tumor specimens, cell processing and flow cytometric analysis

Glioma biopsies of fresh tumor tissue were obtained from patients operated at the Clinic of Neurosurgery, Lund University Hospital, Sweden. Formaldehyde-fixed, paraffin embedded tissue blocks derived from the same surgical excision biopsies were obtained from the Department of Pathology, Lund University Hospital. Patient survival data were obtained from the Swedish National Register of Population (National Board of Health and Welfare). Permission for using these materials was obtained from The Regional Ethical Review Board in Lund, and written informed consent was obtained from patients.

For preparing viable glioma cells, freshly obtained specimen were cut finely into small pieces, treated in IMDM with 0.5 mg/ml collagenase (Sigma) and 25 μg/ml DNase (Sigma) at 37°C for 40 minutes. Red cells were lysed with NH4Cl; the resulting cells were washed in PBS containing 2% FCS. For flow-cytometry analysis, these cells were either used directly or resuspended in freezing medium containing 10% DMSO and 90% FCS for storage in liquid nitrogen. About 5 to 10x10⁶ of the freshly isolated or thawed cells were first incubated with non-specific blocking mouse IgG at 50 μg/ml (clone MOPC 21, Sigma) at 4°C for 20 minutes. Subsequently, about 5x10⁵ cells were stained with allophycocyanin (PE)-conjugated anti-CD45 (clone 30-F11), CD133 (clone AC133), or the isotype-matched control antibody at 50 μg/ml (clone R1), CD44 (clone G44-26), CD24 (clone ML5), EGFR (clone EGFRI) or CD117 (clone AC133), or the isotype-matched control mAbs, at saturating concentrations at 4°C for 15 minutes. Subsequently, cells were washed once with PBS and resuspended in 500 μl PBS supplemented with 2% FCS and 1.0 g/ml 7-aminoactinomycin D (7-AAD, Sigma). The A2B5, O4, and O1 staining was detected using PE-conjugated rat-anti-mouse-IgM (clone 5C3, BD Pharmingen) or PE-conjugated goat-anti-rabbit-IgG (clone 9406, Cell Signaling Technology) or Alexa Fluor 488-conjugated goat-anti-rabbit-IgG (clone 11100, Life Technologies). The A2B5, O4, and O1 staining was detected using PE-conjugated rat-anti-mouse-IgM (clone 5C3, BD Pharmingen) or PE-conjugated goat-anti-rabbit-IgG (clone 9406, Cell Signaling Technology) or Alexa Fluor 488-conjugated goat-anti-rabbit-IgG (clone 11100, Life Technologies).

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A

CD45

PDGFRα  A2B5  O4  CD44

CD24  EGFR  CD133

B

CD45

PDGFRα  A2B5  O4  CD44

CD24  EGFR  CD133

CD133 Marks Poor Survival
when sufficient cells were available. CD133 expression was analyzed with two batches of anti-CD133 mAb with comparable data.

Karyotype analysis of short-term cultured glioma cells

Freshly isolated or thawed glioma cells were cultured in DMDM/F12 medium supplemented with 2% fetal calf serum, basic fibroblast growth factor (20 ng/ml), platelet derived growth factor-AA (20 ng/ml), sonic hedgehog (2 ng/ml) and 1×N2.

Chromosome banding analysis was performed between passage 3 to 4 by standard methods.

Immunohistochemistry analysis

Sections of five-μm thickness were mounted on capillary glass slides (DAKO ChemMate Capillary Gap Microscope Slides, 75 mm, DAKO Sweden AB). All sections were microwave pretreated in 10 mM citrate buffer pH 6.0 for 15 minutes at 800 W in order to achieve antigen retrieval. An automated immunostainer (TechMate™ 500 Plus, DAKO Sweden AB) was used for the staining procedure using DAKO ChemMate Kit Peroxidase/3,3′-diaminobenzidine. Primary antibodies used were GFAP (DAKO, polyclonal, 1:5000 dilution), NSE (Zymed, clone NSE-1G4, 1:1500 dilution) and synaptophysin (DAKO, polyclonal, 1:1000 dilution).

Table 1. Expression of neural stem cell and glial progenitor surface markers in low- and high-grade glioma specimens analyzed.

| Patient ID (Age/gender) | Pathological diagnosis | Cells expressing indicated markers (%) |
|-------------------------|------------------------|----------------------------------------|
|                         |                        | PDGFRa | A2B5 | O4 | O1 | CD44 | CD24 | EGFR | CD133 |
| Low-grade glioma        |                        |        |      |    |    |      |      |      |       |
| 1 (45/F)                | oligoastrocytoma (II)  | 89     | 97   | 99 | 13 | 97   | 99   | UD    | 2.5   |
| 2 (6/M)                 | pilocytic astrocytoma  | 100    | ND   | ND | ND | 100  | UD   | UD    |       |
| 3 (7/F)                 | pilocytic astrocytoma  | 78     | 59   | 96 | ND | 89   | UD   | 15    |       |
| 4 (36/M)                | astrocytoma (II)       | 71     | 100  | 100| 11 | 78   | 78   | 71    | 8     |
| 5 (52/M)                | astrocytoma (II)       | 75     | 79   | 84 | 43 | 76   | 30   | 28    | 8     |
| 6 (60/M)                | astrocytoma (II)       | UD     | 100  | 100| 29 | 100  | 44   | UD    |       |
| High-grade glioma       |                        |        |      |    |    |      |      |      |       |
| 7 (47/M)                | GBM                    | UD     | ND   | ND | ND | 100  | ND   | UD    | UD    |
| 8 (31/M)                | anaplastic oligodendroglia | UD   | 100  | 100| 18 | 61   | UD   | UD    | UD    |
| 9 (62/M)                | GBM                    | UD     | 50   | UD | ND | 100  | UD   | 30    | UD    |
| 10 (8/M)                | anaplastic astrocytoma | 32     | 43   | 45 | 32 | 32   | UD   | UD    | UD    |
| 11 (66/M)               | GBM                    | UD     | 46   | 17 | 13 | 100  | UD   | UD    | UD    |
| 12 (36/M)               | GBM                    | 45     | 64   | 58 | ND | 26   | 12   | 27    | 10    |
| 13 (70/F)               | GBM                    | UD     | 79   | 19 | ND | 96   | 9    | UD    | 72    |
| 14 (47/F)               | GBM                    | 63     | 77   | 90 | ND | 92   | UD   | 92    | 77    |
| 15* (38/M)              | GBM                    | 96     | 95   | 97 | 66 | 90   | 93   | 80    | 29    |
| 16* (49/M)              | oligoastrocytoma III   | 98     | 96   | 96 | 64 | 99   | 98   | UD    | 22    |
| 17 (65/M)               | GBM                    | 69     | 100  | 100| 26 | 85   | 24   | 96    | 90    |
| 18 (51/F)               | GBM                    | 30     | 63   | 74 | ND | 87   | UD   | 83    | 16    |
| 19 (63/F)               | GBM                    | UD     | ND   | ND | ND | 83   | ND   | 73    | 70    |
| 20 (67/M)               | GBM                    | 50     | ND   | ND | ND | 100  | UD   | 100   | 50    |
| 21 (58/F)               | GBM                    | 38     | 74   | 79 | 46 | 88   | UD   | 50    | 53    |
| 22 (61/F)               | anaplastic oligodendroglia | 100  | 100  | 100| ND | 100  | 100  | 100   | 54    |
| 23 (55/F)               | GBM                    | 38     | 85   | 94 | 83 | 81   | 66   | 72    | 47    |
| Other types of brain tumors |                     |        |      |    |    |      |      |      |       |
| 24 (45/M)               | PNET                   | UD     | UD   | UD | UD | UD   | UD   | UD    | 94    |
| 25 (62/M(24) (45/M)     | anaplastic ependymoma  | 25     | UD   | UD | UD | 100  | UD   | 100   | 15    |

Freshly prepared or thawed glioma cells were first incubated with non-specific mouse IgG1 mAb, cells were subsequently incubated with APC conjugated anti-CD45 mAb in combination with FITC conjugated anti-CD44, or anti-CD24 mAbs or PE conjugated anti-PDGFRα, anti-EGFR, anti-CD133 mAbs. For A2B5 and O4 staining, cells were incubated with unconjugated A2B5 or O4 mAb respectively and subsequently stained with PE conjugated rat anti-mouse IgM. Cells negatively stained with 7-AAD were analyzed for cell surface marker expression. Data shown are the percentages of CD45 negative cells positively stained for indicated cell surface markers.

*: secondary GBM. UD: undetectable; ND: not done. PNET: primitive neuroectodermal tumor.

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GFAP and NSE + specimens, CD133 expression was observed at lower frequencies, (Table 2 and Figure 2), suggest that low-grade glioma cells are GFAP staining and the absence of NSE/synaptophysin staining analyzed (Table 1). These data, combined with the homogenous negative cells. The concomitant expression of these progenitor cell markers was observed in nearly all low-grade glioma specimens analyzed (Table 1). These data, combined with the homogenous GFAP staining and the absence of NSE/synaptophysin staining (Table 2 and Figure 2), suggest that low-grade glioma cells are reminiscent of glial-progenitor cells. In 4 out of 6 low-grade glioma specimens, CD133 expression was observed at lower frequencies, but these CD133+ cells are predominantly of blood vessel endothelial origin (see below). The expression of EGFR was found in specimens from 2 of the 6 low-grade gliomas. We also analyzed the expression of CD24. Absence of CD24 expression in combination with positive staining on CD133, or PNA or ABCG2/Bcrp1 was used to enrich primitive neural cells with “stem cell-like” properties [17,35–38]. CD24 expression was ABG2/Bcrp1 was used to enrich primitive neural cells with “stem cell-like” properties [17,35–38]. CD24 expression was derived from newly formed blood vessel endothelial cells, and not from the glioma cells. In GBM specimens, the majority of CD133+ cells were of glioma origin, although CD31+/CD133+ cells (ranging from 0.5 to 10% of the total living cells, n = 9) were also found. In agreement with previous studies demonstrating glioma expression of oligodendrocyte progenitor cell transcription factor Olig1/2 and surface antigen NG2 [1–3], the concomitant expression of A2B5 (a marker for multiple types of progenitor cells in glial lineage [20–27,41]), PDGFRα (a marker for early oligodendrocyte progenitor cells at O-2A stage [22,25,26,42–44]) and O4 (a marker for late oligodendrocyte progenitor cells [29]) suggests that glioma cells, irrespective of their morphological characteristics, are committed to the oligodendrocyte lineage. To further characterize the differentiation stages along the oligodendrocyte lineage downstream of the A2B5 expressing glial progenitor cell level, we analyzed the expression of O1, a marker for pre-myelinating oligodendrocytes [29]. In contrast to the high level A2B5 and O4 expression, the frequency of O1+ cells were significantly diminished in all low-grade and the majority of GBM specimens analyzed (Table 1 and Figure 5). The expression of GFAP and these oligodendrocyte progenitor surface markers suggests that along the oligodendrocyte lineage differentiation hierarchy, A2B5+/PDGFRα+ glioma cells were

| Table 2. Co-expression of neuronal and glial markers in high-grade glioma cells |
|-----------------|-----------------|
| Patient ID      | GFAP | NSE   |
| 1 (45/F)        | ++   | -     |
| 2 (6/M)         | ++   | -     |
| 3 (7/F)         | ++   | -     |
| 4 (36/M)        | ++   | -     |
| 5 (52/M)        | ++   | -     |
| 6 (60/M)        | ++   | -     |
| 7 (47/M)        | +++  | +     |
| 8 (31/M)        | ++   | -     |
| 9 (62/M)        | +++  | ++    |
| 10 (8/M)        | +++  | +     |
| 11 (66/M)       | +++  | ++    |
| 12 (36/M)       | +++  | +     |
| 13 (70/F)       | ++   | +     |
| 14 (47/F)       | +++  | +     |
| 15 (38/M)       | +++  | +     |
| 16 (49/M)       | ++   | +     |
| 17 (65/M)       | ++   | +     |
| 18 (51/F)       | ++   | +     |
| 19 (63/F)       | +++  | +     |
| 20 (67/M)       | +++  | +     |
| 21 (60/M)       | +++  | +     |
| 22 (61/F)       | ++   | +     |
| 23 (53/F)       | +++  | +     |
| 24 (45/M)       | +    | +     |
| 25 (62/M)       | ++   | -     |

The GFAP and NSE staining was performed in the fixed glioma specimens from the same surgical procedures as those used for generating viable cells. The staining was semi-quantitatively evaluated: +++, markedly positive staining; ++, moderately positive staining; +, low positive; - , no positive staining in the tumor. doi:10.1371/journal.pone.0001936.t002
compromised in differentiation downstream of A2B5/O4, but upstream of the O1 stage.

To study glioma cell differentiation potential along neuronal lineage, we performed an immunohistochemical staining for pan-neuronal marker NSE and synaptophysin expression in archival specimens (Table 2 and Figure 2). In low-grade gliomas, no obvious expression of NSE and synaptophysin was detected, but the cells homogeneously expressed GFAP (Table 2 and Figure 2). We were unable to ascertain whether the few cells weakly stained for NSE were the intermingled normal neuronal cells or the glioma cells (Figure 2, upper panel). In high-grade glioma cells, a regional NSE expression was clear in all cases analyzed (Table 2 and Figure 2), although both region-dependent and homogeneous expression patterns were detected. Synaptophysin positivity was detected in 7 high-grade gliomas. These data, in combination with the expression of GFAP and multiple oligodendrocyte lineage progenitor surface markers (Tables 1 and 2, Figures 1, 2 and 3), suggest that compared to low-grade glioma cells, high-grade glioma cells maintain glial progenitor-like features, but additionally exhibit enhanced CD133 expression as well as neuronal differentiation potential.

Transformed glial progenitor-like cells can provide a niche environment recruiting normal stem cells or progenitor cells into the tumor mass [45]. To investigate whether freshly isolated glioma cells studied here indeed contained transformed cells, karyotype analysis was performed in short-term cultured glioma cells (Table 3). We only observed normal karyotype for cells derived from patients 3 and 8. Abnormal karyotypes were observed from cells derived from patients 4, 12, 13, 14, 17, 18 and 20. Of note, cultures derived from low-grade glioma patient 4 and high-grade glioma patient 12 contained about 40% of the cells with a normal karyotype, contrasting the nearly homogenous phenotype between all cells (Figures 1, 3); but cultures derived from high-grade glioma patients 13, 14, 17, 18 and 20 predominantly contained cells with abnormal karyotypes. In addition, fresh cells from patients 14 and 20 formed xenograft glioma following subcutaneous injection into SCID mice [46]. Thus, the analyzed glioma cells did contain neoplastic cells and the karyotype normal and abnormal glioma cells showed hitherto indistinguishable phenotype.

Finally, we investigated whether the glial progenitor or stem cell markers expressed on glioma cells could serve as prognostic
Figure 3. Maintenance of glial progenitor-like phenotype, but enhanced CD133 expression in high-grade gliomas. In addition to multiple glial progenitor cell markers, a high proportion of glioma cells co-expressing CD133 was detected in most of the high-grade glioma specimens. Dot-plot profiles of glial progenitor cell surface markers and CD133 expression on high-grade glioma cells from representative patients (#14 (A) and #22 (B)) are shown. Freshly isolated glioma cells were simultaneously stained with the indicated antibodies. The hematopoietic cells were distinguished with anti-CD45 staining. The numbers in each quadrate represent the percentages of the cells stained positively or negatively by the respective antibodies.

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Figure 4. Vessel or glioma origin of CD133 expressing cells. In contrast to high-grade glioma specimens, CD133 expressing cells detected in low-grade glioma specimens are predominantly derived from blood vessel endothelial cells. Dot-plot profiles of CD133 expression versus CD45 and/or CD31 expression of cells from glioma specimens of indicated patients are shown. The numbers in each quadrate represent the percentages of the cells stained positively or negatively by the respective antibodies.
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markers for patient survival. Glioblastoma patients receiving immune therapy and patients with pilocytic astrocytoma or ependymoma, were excluded from the prognosis analysis. We divided all analyzed grade II to IV glioma patients into a CD133\(^+\) low group (CD133\(^+\) cells less than 30\%) and a CD133\(^+\) high group (CD133\(^+\) cells higher than 30\%). The median survival time in the CD133\(^+\) high group was 5.0 \(\pm\) 6.9 months (\(n = 9\)) compared to more than 22.0 \(\pm\) 17.3 months (\(n = 10\)) in the CD133\(^+\) low group \((P = 0.026, t\) test; Figure 6). Thus, CD133 expression inversely correlates with glioma patient survival time.

**Discussion**

In this study, we investigated the expression of multiple cell surface markers characteristic of glial genesis hierarchy in freshly isolated viable glioma cells as well as the expression of GFAP, NSE and synaptophysin in the fixed specimens from a cohort of low-grade and high-grade glioma patients. Our data show that nearly all low-grade glioma cells from the analyzed cases concomitantly express multiple cell surface markers for glial progenitor cells, such as PDGFR\(\alpha\), A2B5, O4, and CD44. The expression of these glial progenitor markers is maintained in the high-grade gliomas analyzed. GFAP expression was detected, as anticipated, in all low- and high-grade gliomas analyzed, but the expression of NSE and synaptophysin was only detected in high-grade gliomas. Co-staining of CD31 suggests that the rare CD133 expressing cells in low-grade gliomas are predominantly of vessel endothelial origin. In more than 50\% of the high-grade glioma cases, distinct CD133 expression was detected in cells concomitantly expressing multiple glial progenitor markers. Thus, low-grade glioma cells may resemble the expansion of A2B5\(^+\) glial progenitor-like cells; high-grade gliomas, in addition to the expansion of A2B5\(^+\) glial
progenitor-like cells, may also contain CD133\(^+\) putative glioma stem cells that concomitantly express multiple surface markers of glial progenitor cells. Irrespective of CD133 expression, high-grade glioma cells are endowed with multi-lineage differentiation potential in vivo. Importantly, CD133 expression negatively correlated with the glioma patient survival time.

Conserved mechanisms across organ development/homeostasis and tumorigenesis have been demonstrated in many types of human cancers [47]. It is likely that gliomas may originate from the primitive cells along the postnatal glial genealogy hierarchy. In fact, glial progenitor cells were demonstrated to be more susceptible to oncogene transformation compared to mature astrocytes [48]. Glial genealogy originates from neural stem cells via multiple types of intermediate glial precursors [49]. In adult human CNS, putative neural stem cells have been identified in restricted brain regions [50]. In contrast, A2B5 and PDGFR\(\alpha\) expressing O-2A progenitors are relatively abundant throughout the CNS [24,25,41,51–53]. The aforementioned A2B5\(^+\) "PDGFR\(\alpha\) GRPs and A2B5\(^+\)PDGFR\(\alpha\) CD144 ARPs may also exist. In addition to well-established PDGFR\(\alpha\) expression, we identified that the majority of low-grade astrocytomas and high-grade gliomas concomitantly express multiple markers of glial progenitor cells such as A2B5 and O4. Our findings seem not consistent with previous reports that astrocytomas could be divided into A2B5\(^+\) and A2B5\(^-\) lineages [34,55]. This discrepancy is likely caused by the fact that the epitopes recognized by the A2B5, O4 and O1 antibodies are not well maintained in formalin fixed, paraffin embedded specimens, and our studies were performed in living glioma cells. It is unlikely that the co-expression of these cell surface markers is a consequence of a cell type dependent/independent program inherent to all glioma cells, rather it reflects the differentiation stage(s) common to normal glial genealogy hierarchy and glioma cells. In line with previous reports identifying oligodendrogial lineage markers such as Olig2 or NG2/PDGFR\(\alpha\) as universal markers in diffuse gliomas [1–3], our study demonstrates the shared pattern of concomitant expression of multiple surface markers between glioma cells and oligodendrocyte progenitor cells, which suggests that most of the morphologically diagnosed astrocytoma and GBM are endowed with oligodendrocyte lineage differentiation potential. The data of weak O1 expression, but homogenous GFAP expression, indicate that these cells are likely blocked at differentiation pathways between O4 and O1 stages in oligodendrocyte lineage or at hitherto unidentified stages towards type-2 astrocytes [20,21,28].

The expression of CD144 identifies the ARPs [28] and nearly all gliomas express this marker. This CD144 expression does not necessarily contradict the oligodendrocyte lineage commitment of A2B5\(^+\)PDGFR\(\alpha\)O4\(^+\) glioma cells which exhibited a concomitant GFAP expression, because a misexpression of CD144 can result in expansion of oligodendrocyte progenitor cells with impaired maturation and concomitant gain of GFAP expression [28]. However, it cannot be excluded that A2B5\(^-\)CD144\(^+\)GFAP\(^-\) glioma cells without detectable PDGFR\(\alpha\) and O4 expression (e.g.: case \#9, Table 1) actually represent the expansion of ARP-like cells.

Previous neurosphere culture-based studies have demonstrated that sphere-forming glioma cells are capable of multi-lineage differentiation [8,9,56,57]. A potential inherent weakness in this type of studies, however, is that neurosphere culture studies only assess the particular fractions of glioma cells, which are supported by the neurosphere culture conditions [10], and the neurosphere culture conditions do not necessarily represent the glioma niche environment. Additionally, multi-lineage differentiation capacity can also reflect the differentiation plasticity induced by neurosphere culture conditions. It has been demonstrated that lineage-restricted progenitors gain multi-lineage differentiation capacity in neurosphere-forming assay [11,58]. We demonstrate that high-grade glioma cells, in contrast to low-grade glioma cells, can express neuronal marker as well as CD133 in vivo. Thus, our data suggest that the in vivo multi-lineage differentiation capacity is likely restricted to high-grade glioma cells. It is unclear whether the in vivo multi-lineage differentiation capacity can be gained following the progression from low-grade to high-grade gliomas.

Gliomas are composed of a mixture of neoplastic and non-neoplastic cells [45]. The non-neoplastic cells could include entrapped neurons, astrocytes, microglial cells, blood vessel cells. It is also demonstrated in mouse glioma models that normal progenitor or stem cells can be recruited to glioma niche [45]. Although the possibility could not be entirely excluded that the cells with normal karyotype or isolated numerical aberrations are also neoplastic, our data suggest that most of the entrapped cells in human gliomas show a progenitor or stem cell-like phenotype and such cells can constitute more than 40% of the total cells in low-grade gliomas, but their frequency varies substantially in GBM specimens. It remains to be established whether neoplastic and non-neoplastic cells in gliomas can be phenotypically distinguished.

Considering gliomas as a group of progressive tumors, our data demonstrate that expression of the CD133 associated stem cell features is correlated with a poor prognosis. High-grade glioma patients without detectable frequencies of CD133 expressing cells as well as grade II glioma patients appeared to survive significantly longer compared to high-grade glioma patients with high frequencies of CD133 expressing glioma cells. This finding extends previous studies that “stem cell-ness” gene expression

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Table 3. Karyotype analysis of short-term cultured glioma cells

| Patient ID | Karyotype |
|------------|-----------|
| **Low-grade glioma** | |
| 3 | normal |
| 4 | 45,X,Y[13]/46,XY[10] |
| **High-grade glioma** | |
| 8 | normal |
| 12 | 45,X,Y[11]/46,XY,Y,+7/33/46,XY[10] |
| 13 | 41-42,X,Y,der(1;17)(p36;q21),add(4)(q31), del(6)(q11),-7,-10,-11,13,14,17,del(14)(q22),-15, -17, +2mar,inc(cp9)/80-84, idem x2 (cp16) |
| 14 | 44-45,X,Y,der(7)(del(7)(q35),-10,-13,del(14)(q22), del(16)(q21)(cp9)/43,X,Y,der(1;14)(p34;q22),-4, der(6)(1;7)(q23;p11),+der(7)(7)(q35);der(8)(8;9) |
| 17 | 45,X,Y[14]/46,XY,Y,+7/33/46,XY[10] |
| 18 | 44,XX,y,del(9;16)(p23;q12);del(10)(p12),del(12) t(12;14)(q13;q12);-13,-14;del(16)(q21,der(17)(16t(17)(q11), |
| 20 | -17, +2mar,inc(cp9)/80-84, idem x2 (cp16) |

Freshly isolated glioma cells were cultured and analyzed for karyotype between passage 3 to 4. The number in brackets represents the number of the indicated karyotype.

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pattern can serve as a marker for poor prognosis of malignant diseases [59,60]. Our findings are consistent with a recent report on a correlation between enhanced CD133 expression and a poor clinical outcome in glioma patients [61]. Importantly, we show that the rare CD133 expressing cells in low-grade gliomas are predominantly of blood vessel origin. Of note, the frequencies of CD133 expressing cells in high-grade gliomas analyzed in our studies appeared to be much higher compared to previous reports [7,56,61]. This discrepancy could be because we analyzed the CD133 expressing cells in CD45-negative cell fraction and glioma specimens can have different contents of hematopoietic cells.

This study constitutes an important step towards clarifying the lineage commitment and differentiation blockade of glioma cells. We have demonstrated that normal glial progenitor cell surface markers are widely expressed in glioma cells. Glial progenitor cell surface markers could potentially be used to design glial progenitor antigen-targeted immune therapy or gene therapy. As normal neural stem cells are believed to be devoid of glial progenitor cell surface markers, targeted delivery of cytotoxic agents to selectively ablate glioma cells would spare the normal neural stem cells.

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Author Contributions

Conceived and designed the experiments: XF. Performed the experiments: DG XF JR DT AP EE. Analyzed the data: DG XF JR DT BW LS AP EE. Contributed reagents/materials/analysis tools: LS. Wrote the paper: DG XF JR DT BW LS AP EE.

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