

Aggressive invasion is observed in CD133⁻/A2B5⁺ glioma-initiating cells

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Abstract. Glioblastoma multiforme is the most common and fatal primary brain tumor in adults. Aggressive invasion of glioblastoma cells into brain tissue often limits complete surgical resection and contributes to therapeutic resistance. The cell surface marker, CD133, has been identified as a putative stem cell marker in normal and malignant brain tissues; CD133⁻/A2B5⁺ cells exhibit neural stem-like cell properties. The invasive properties and the molecular mechanisms of CD133⁻/A2B5⁺ glioma-initiating cells (GICs) were investigated in the process of self-renewal and tumorigenesis. An increased number of invasive cells through matrigel and an increase in migratory cells through filters were observed in CD133⁻/A2B5⁺ GIC populations compared with matched non-initiating tumor cell populations. Considerable changes were detected in expression of mRNA and protein associated with migration or invasion. CD133⁻/A2B5⁺ GIC demonstrated infiltrating growth patterns and displayed greater invasive potential under fluorescent microscopy comparing with the matched non-initiating tumor cells after cells labeled with red fluorescence protein were transplanted into the brains of athymic/nude mice. CD133⁻/A2B5⁺ GICs possess strong migratory and invasive capacity. These infiltrating cells in the invasive fronts may be responsible for rapid tumor recurrence following conventional treatments. CD133⁻/A2B5⁺ GICs may be an important subpopulation with high invasive potential and they should not be ignored when targeting GICs to prevent GBM recurrence.

Introduction

Glioblastoma multiforme (GBM) is the most common and fatal primary brain tumor in adults. Despite available treatments, including surgical resection, chemotherapy and radiotherapy, the vast majority of individuals only survive 1-2 years following diagnosis (1). Poor prognosis is attributed to extensive infiltration to brain tissue and intrinsic resistance to conventional treatments. Aggressive invasion of GBM cells into brain tissue often limits complete surgical resection and contributes to therapeutic resistance, which results in fatal tumor recurrence (2). Thus, the development of strategies targeting the invading cells or restraining their invasive capacity is likely to provide effective therapies.

Increasing evidence has supported the hypothesis that a rare subpopulation of cancer cells sharing stem cell characteristics within a GBM has potent capacity of tumor propagation (3,4). These cells are termed glioma stem cells (GSCs); they demonstrate greater tumorigenic potential compared with matched non-stem tumor cells when xenotransplanted into the brains of immunocompromised rodents (5-8). The work of Singh *et al* (9) firmly established the existence of a subpopulation of cells with a 'stem cell-like' phenotype, expressing CD133 cell surface marker, within malignant brain tumors. CD133 and nestin are currently the most accepted markers for identification of GSCs. However, certain studies have proposed that there is not a hierarchical association between CD133⁺ and CD133⁻ cells composing neurospheres (10).

A2B5 antigen is recognized as a marker of neural progenitor cells, and explants from A2B5⁺ tumor cells displayed a typical progenitor morphology and clearly indicated their immature state (11). In a previous study, the majority of A2B5⁺ multipotential progenitor cells differentiated to oligodendrocytes and a minority of these cells differentiated to neurons (12). A2B5⁺ cells exhibit the potential to differentiate into oligodendrocytes and type-1 and type-2 astrocytes, and all xenografts containing A2B5⁺ cells generated migrating cells with distinctive functional properties according to glioma subtypes (11). The A2B5⁺ cells, but not the A2B5⁻ cells isolated from GBM, have neural stem-like cell properties. Thus the A2B5⁺ initiating cells may be sorted into two populations, the A2B5⁺/CD133⁺ and A2B5⁺/CD133⁻ cells, according to expression of CD133 antigen.

At present, there have been no studies directly examining the migratory and invasive potential of glioma-initiating cells

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(GICs), expressing CD133⁺/A2B5⁺ surface markers, compared with matched differentiated cells. The invasive potential of CD133⁺/A2B5⁺ GICs and differentiated non-initiating tumor cells were investigated *in vitro* and *in vivo* in a mouse tumor xenograft model.

Materials and methods

GICs culture. Tumor tissues from a human GBM surgical specimen, which were collected from a 23-year-old man in The First Affiliated Hospital of Soochow University (Suzhou, China), were washed, deprived of vessels, acutely dissociated in phosphate buffered saline (PBS) and subjected to enzymatic dissociation. The patient provided written informed consent to participate in the study, which was approved by the Ethics Review Board of the First Affiliated Hospital of Soochow University (no. 2012070). Cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) for ~2 months, then the glioma cells were subsequently placed in serum-free DMEM/F12 medium supplemented with 1% N2 (Gibco; Thermo Fisher Scientific Waltham, MA, USA), 20 ng/ml epidermal growth factor [EGF (Invitrogen; Thermo Fisher Scientific)], and 20 ng/ml basic fibroblast growth factor [bFGF (Invitrogen; Thermo Fisher Scientific)] for ~7 days and formed non-adhesive neurospheres. Neurospheres were maintained by changing half of the medium every 3 days and collected by centrifugation at 1,000 × g for 10 min. Subspheres were formed for 3~4 days after primary spheres were dissociated mechanically to single cell suspension. Neurospheres of ~12 passages were used for sorting. Magnetic isolation of CD133⁺/A2B5⁺ GICs population was performed using the Miltenyi Biotec A2B5 and CD133 Cell Isolation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Cells were cultured at 37°C in a humidified 5% CO₂/95% air atmosphere.

Immunofluorescence staining for GICs markers. Tumor spheres were plated onto poly-L-lysine-coated glass coverslips. Cells were fixed with 4% paraformaldehyde for 20 min at room temperature, washed three times with PBS, blocked with 2% goat serum (Boster Bio-Engineering, Wuhan, China) for 30 min and permeabilized with 0.1% Triton X-100 (Beyotime Institute of Biotechnology, Shanghai, China). Cells were incubated with primary monoclonal anti-human mouse CD133 (1:200; cat. no. 130-090-422; Miltenyi Biotec GmbH), monoclonal anti-human rabbit nestin (1:300; cat. no. ab105389; Abcam, Cambridge, MA, USA) and monoclonal anti-human mouse A2B5 (1:200; cat. no. ab53521; Abcam) antibodies overnight at 4°C. Subsequently, cells were washed three times with PBS. Secondary anti-mouse IgGs conjugated with Alexa fluor 555 (1:1,000; cat. no. A-21422; Molecular Probes; Thermo Fisher Scientific) or anti-rabbit IgGs conjugated with Alexa fluor 488 (1:1,000; cat. no. A-11008; Molecular Probes; Thermo Fisher Scientific) were used in darkness for 30 min at room temperature. The nuclei were counterstained with anti-fade sealant containing 4',6-diamidino-2-phenylindole (DAPI) (5 µg/ml; SouthernBiotech, Birmingham, AL, USA). Fluorescence images were captured using a fluorescence microscope (Olympus BX40; Olympus Corporation, Tokyo, Japan).

Flow cytometric analysis of CD133⁺/A2B5⁺ cells. GICs were dissociated into single cell suspension, then incubated with PE-conjugated anti-human mouse IgG A2B5 antibody (1:100; Miltenyi Biotec GmbH) and APC-conjugated anti-human mouse IgG CD133 antibody (1:100; Miltenyi Biotec GmbH). Labeled cells were analyzed by a flow cytometer Beckton Dickinson FACScan (BD Biosciences, San Jose, CA, USA) (13). Unsorted GICs were also analyzed as a control.

Differentiation of GICs into non-initiating tumor cells. GICs were differentiated into non-initiating tumor cells in a high glucose DMEM of 10% FBS without N2/EGF/bFGF for 28 days. The cells were blocked with normal goat serum for 30 min. Next, the cells were incubated with monoclonal anti-human rabbit SOX2 (GSC marker; 1:200; cat. no. ab97959; Abcam), monoclonal anti-human mouse Tuj1 (neuron marker; 1:200; cat. no. ab14545; Abcam), monoclonal anti-human mouse GFAP (astrocyte marker; 1:300; cat. no. ab10062; Abcam) and monoclonal anti-human mouse Galc (oligodendrocyte marker; 1:100; cat. no. ab125086; Abcam) primary antibodies overnight at 4°C. Secondary anti-mouse IgGs conjugated with Alexa fluor 555 (1:1,000; cat. no. A-21422; Molecular Probes; Thermo Fisher Scientific) or anti-rabbit IgGs conjugated with Alexa fluor 488 (1:1,000; cat. no. A-11008; Molecular Probes; Thermo Fisher Scientific) were used in darkness for 30 min at room temperature. The nuclei were counterstained with DAPI (5 µg/ml). Fluorescence images were captured using a fluorescence microscope (Olympus BX40; Olympus Corporation) to confirm that differentiation had occurred.

***In vitro* cell migration and matrigel invasion assay.** The *in vitro* cell invasion assay was performed using a Matrigel-coated (3 mg/ml) invasion chamber (BD Biosciences), while the migration assay was performed by control inserts. Cell culture medium supplemented with 10% FBS was added to the lower chamber to act as a chemoattractant. Cells were seeded at a density of 1×10⁵ cells/well onto the upper inserts. After incubation for 24 h at 37°C, the non-migratory or non-invasive cells were removed from the upper side of the filter by gentle scrubbing with a moist cotton swab. The migrating or invading cells in the reverse side of the filter were fixed in 100% methanol and stained with crystal violet (Beyotime Institute of Biotechnology), then counted under an inverted microscope (Olympus CKX41; Olympus Corporation) in 6 fields at random for analysis.

Red fluorescence labeling of cells. Cells were transfected with red fluorescence protein (RFP) gene using a lentivirus mediated gene transfection kit (Shanghai GenePharma Co., Ltd., Shanghai, China) according to the manufacturer's instructions. More than 90% of GICs and non-initiating tumor cells expressed RFP under fluorescence microscope (Olympus IX51; Olympus Corporation), the RFP gene integrated stably in target cell genome and maintained a stable RFP expression in the transfected tumor cells. Invasive or migratory potential of GICs-RFP and non-initiating tumor cells-RFP cells *in vitro* was detected by matrigel assay or polycarbonate filter, respectively, and was observed directly

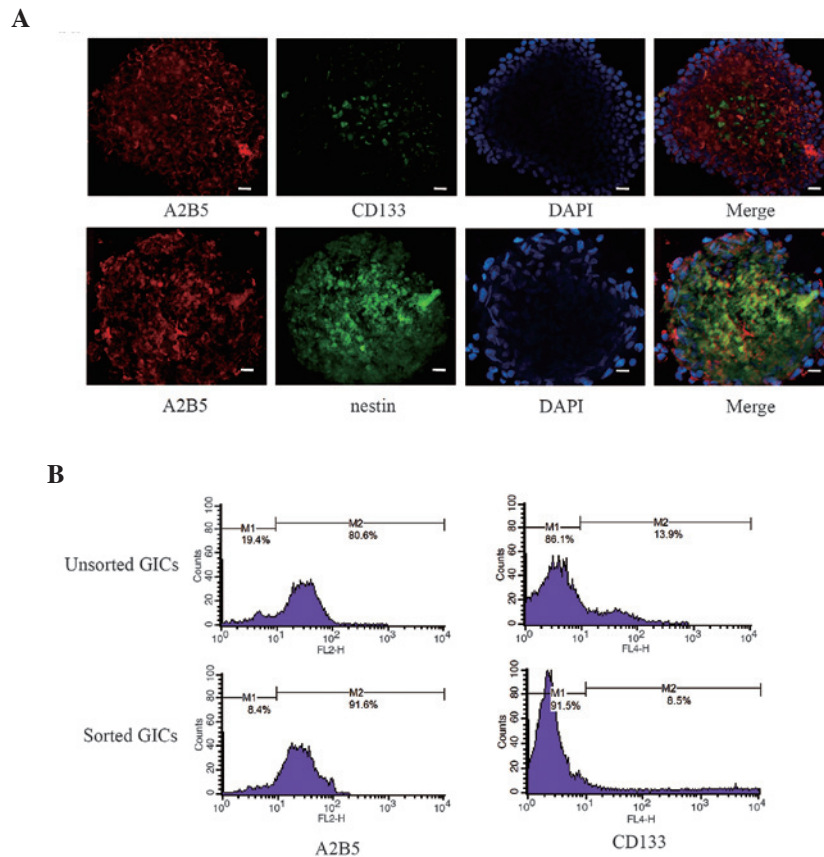


Figure 1. Identification of GICs. (A) Sorted GICs were immunostained with antibodies against CD133, nestin and A2B5 markers. Fluorescence images were captured with fluorescence microscope (x400). Scale bar=20 μ m. (B) The percentage of CD133⁺ and A2B5⁺ cells in unsorted or sorted GICs analyzed by flow cytometry. DAPI, 4',6-diamidino-2-phenylindole.

under fluorescence microscope (Olympus IX51; Olympus Corporation).

Reverse transcription-quantitative polymerase chain reaction (RT-PCR). A total of 3×10^5 cells/well were seeded into 6-well plates in triplicate and incubated at 37°C for 24 h. The cells were lysed, and total RNA was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific) according to the manufacturer's instructions. First strand cDNA synthesis was performed using reverse transcriptase and random hexanucleotide primers (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The complementary DNA was subsequently used to perform qPCR on a LightCycler480 Thermal Cycler (Roche Diagnostics, Basel, Switzerland) using SYBR Green (Molecular Probes; Thermo Fisher Scientific) with gene-specific primers (Gene Pharma, Shanghai, China) and JumpStart™ Taq DNA polymerase (Invitrogen; Thermo Fisher Scientific). The crossing threshold value was normalized to GAPDH, and quantitative changes in mRNA were expressed as fold-change relative to the control \pm standard error of the mean (SEM) value.

Western blot analysis. Cell extracts were prepared by lysing cells in RIPA buffer containing a mixture of protease and phosphatase inhibitor cocktails (Thermo Fisher Scientific, Inc.) followed by sonication and centrifugation at 10,000 \times g for 10 min at 4°C. Protein concentration was determined using

a BCA protein assay (Pierce Biotechnology, Inc., Rockford, IL, USA). A total of 50 μ g of protein was loaded onto a 10% SDS-PAGE gel and transferred to nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked in 5% non-fat dry milk for 1 h and probed overnight with primary antibodies at 4°C. Subsequently the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature. The blots were visualized using enhanced chemiluminescence (ECL) reagents (Invitrogen; Thermo Fisher Scientific) and densitometric quantification was analyzed using Launch Sensi Ansys software (Shanghai Peiqing Science & Technology Co., Ltd., Shanghai, China). The primary antibodies including anti-epithelial cadherin (E-cadherin, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-intercellular adhesion molecule 1 (ICAM-1, Santa Cruz Biotechnology, Inc.), anti-matrix metalloproteinase 2 (MMP-2, Abcam), anti-MMP-9 (Abcam) and anti-tissue inhibitor of metalloproteinase 3 (TIMP3, Abcam) were used. β -actin (Santa Cruz Biotechnology, Inc.) expression was evaluated as a control for protein loading.

Intracranial transplantation to establish GBM xenografts. BALB/c mice (Shanghai Laboratory Animal Center, CAS, Shanghai, China) were divided into 2 groups, and each consisted of 8 mice. Animal care and experimental procedures described in the study were performed in accordance with the

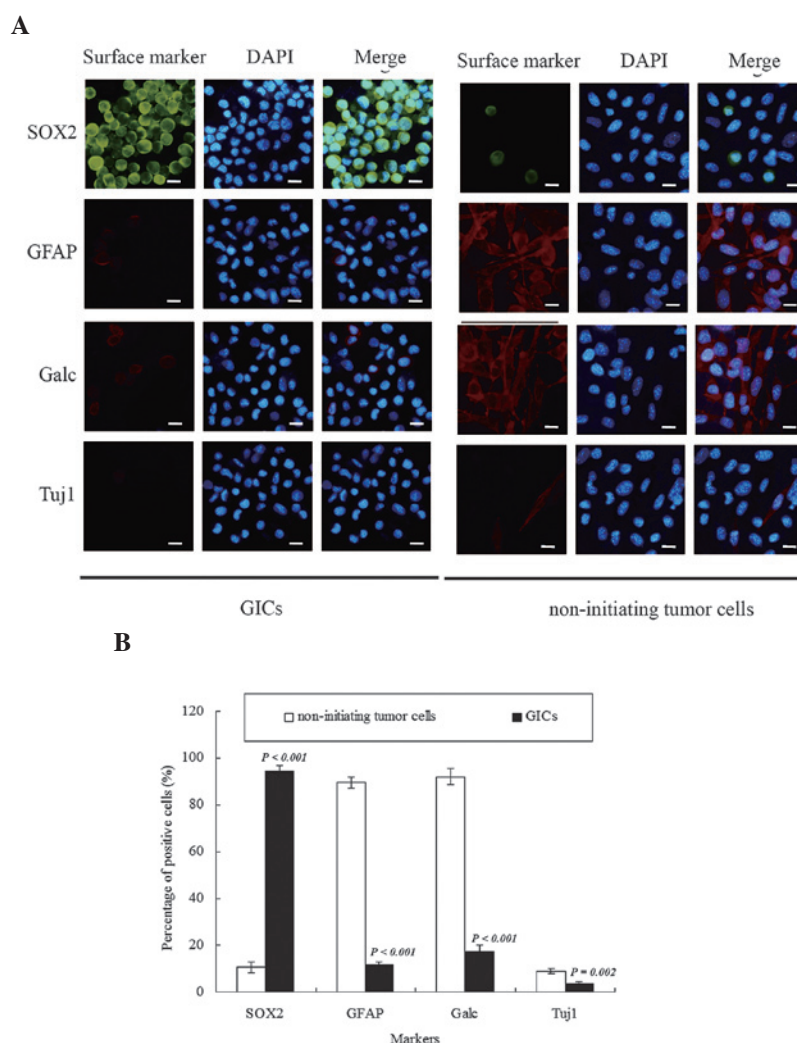


Figure 2. Characterization of GICs and non-initiating tumor cells. (A) High expression of SOX2 and low expression of Tuj1, GFAP and Galc were observed in GICs. In contrast, fewer SOX2 positive cells and more Tuj1, GFAP and Galc positive cells were observed in non-initiating tumor cells (x400). Scale bar=10 μ m. (B) The percentage of positive cells expressing SOX2, GFAP, Galc and Tuj1. GICs, glioma-initiating cells; DAPI, 4',6'-diamidino-2-phenylindole.

Guidelines for Animal Experiments at Soochow University from Institutional Animal Care and Use Committee with the approval of Ethics Committee of the university [Certificate no. SYXK (Su) 2007-0035]. Athymic/nude immunocompromised mice at 6-8 weeks of age were maintained under pathogen-free conditions within the institutional animal facility. Food and water were provided *ad libitum*. Surgical procedures were performed in a sterile fashion. Mice were anesthetized by intraperitoneal injection of chloral hydrate (400 mg/kg), then positioned in a rodent stereotaxic frame. A total of 2×10^5 CD133/A2B5⁺ GICs or matched non-initiating tumor cells were stereotactically injected into the right putamen (1 mm forward, 2 mm right lateral from the bregma, and 2.5 mm down from the dura) by use of a Hamilton syringe to establish GBM xenografts. The mice were sacrificed when symptoms of brain tumor, including sustained weight loss, ataxia and periorbital bleeding, were observed. Prior to the collection of mouse brains bearing GBM tumors, cardiac perfusion with PBS followed by perfusion with 4% paraformaldehyde was performed. The whole brain was harvested and continuously sectioned at a thickness of 5 μ m, then either stained with

hematoxylin and eosin (H&E; Beyotime Institute of Biotechnology) using routine histopathological procedures, or directly observed under fluorescent microscopy. Red fluorescence indicated the presence of tumor cells, whereas normal brain cells exhibited no fluorescence.

Statistical analysis. All determinations were performed from ≥ 3 independent experiments as indicated. The data are presented as the mean \pm SE. Statistically significant differences in mean values between the 2 groups were tested by Student's *t* test. A value of $P < 0.05$ was considered to indicate a statistically significant difference. All statistical calculations were performed using SPSS software, version 11.0 (SPSS Inc., Chicago, IL, USA).

Results

Identification of GICs. Following magnetic sorting, a majority of GICs were shown to exhibit negative expression of glioma stem cell marker CD133 and positive expression of marker A2B5 (CD133/A2B5⁺ cells), which were involved in the self-renewal and proliferation of stem cells (Fig. 1A). Co-expression

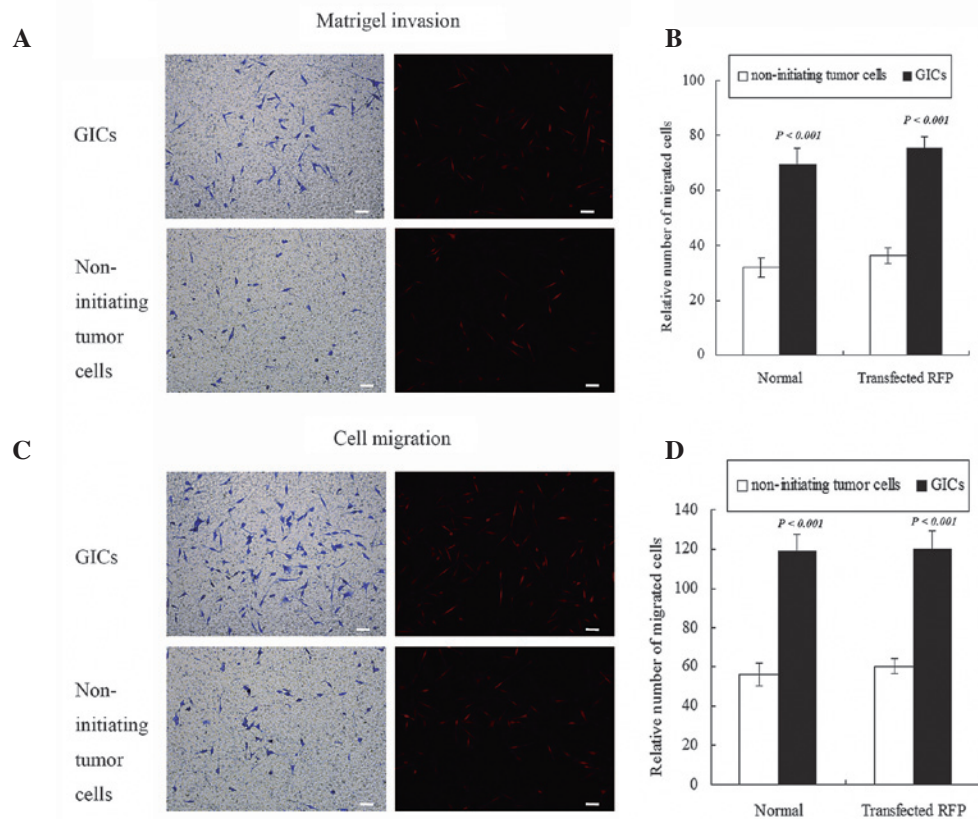


Figure 3. Migration and invasion assay of CD133-/A2B5+ GICs and matched non-initiating tumor cells *in vitro*. Cells passing the matrigel or polycarbonate filters were determined. The number of (A) invading and (C) migrating GICs populations was significantly increased compared with non-initiating tumor cells populations (x100); scale bar=50 μ m. Quantified data of (B) invading and (D) migrating GICs compared with non-initiating tumor cells demonstrated a statistical increase. Both migration and invasion of GICs-RFP or non-initiating tumor cells-RFP, which were transfected with RFP, were not significantly different from the corresponding GICs or non-initiating tumor cells. RFP, red fluorescence protein; GICs, glioma-initiating cells.

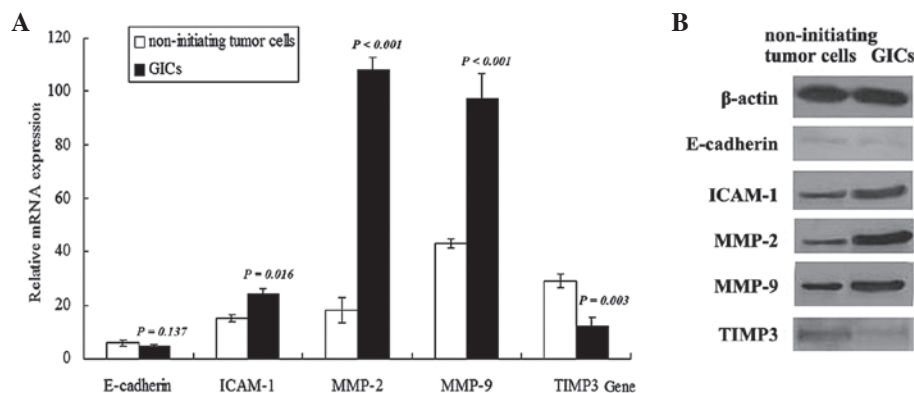


Figure 4. The expression of migration or invasion-associated markers in CD133-/A2B5+ GICs compared with non-initiating tumor cells. (A) The mRNA levels of migration or invasion-associated markers including E-cadherin, ICAM-1, MMP-2, MMP-9 and TIMP3, detected by real-time quantitative RT-PCR, were showed in GICs and non-initiating tumor cells. (B) Associated protein expressions of migration or invasion were observed in GICs and non-initiating tumor cells using western blot analysis. GICs, glioma-initiating cells.

of A2B5 and nestin was observed in the majority of GICs (Fig. 1A). To determine the percentage of cells that expressed the CD133/A2B5⁺ phenotype and the sorting efficiency of the magnetic sorting method, quantitative analysis of CD133 and A2B5 positive cells was performed by flow cytometry. The results demonstrated that the GICs sorted by magnetic beads

consisted of 8.5% CD133⁺ cells and 91.6% A2B5⁺ cells; the unsorted GICs consisted of 13.9% CD133⁺ cells and 80.6% A2B5⁺ cells (Fig. 1B).

Characterization of GICs and differentiated non-initiating tumor cells. A high level of human SOX2, a stemness marker

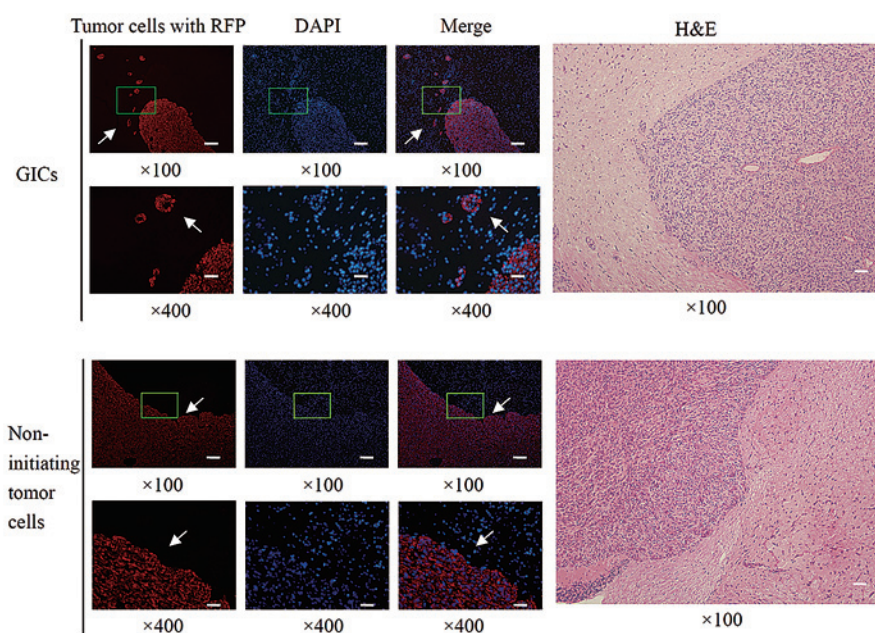


Figure 5. CD133-/A2B5⁺GICs displayed greater invasive capacity compared with matched non-initiating tumor cells *in vivo*. CD133-/A2B5⁺ GICs or non-initiating tumor cells were transplanted into mice brains through intracranial injection. Mice brains after tumor cells transplantation were examined for tumor cells dispersal by fluorescence microscopy. Aggressive invasion of tumor cells (indicated by white arrows) into brain tissues was observed in mice brains implanted with GICs. However, no tumor cells dispersal was observed in the brains implanted with non-initiating tumor cells, and all the cells remained close to the injection site (indicated by a white arrow). Scale bar=160 μ m in fluorescence observation at x100, scale bar=40 μ m in fluorescence observation of x400. The adjacent section pathologically demonstrates the formation of intracranial xenograft tumor. Scale bar=80 μ m in H&E staining. DAPI, 4'6-diamidino-2-phenylindole.

of GSCs, and a low expression level of human Tuj1, GFAP and Galc, differentiation markers of tumor cells, were observed in the GICs. In contrast, a reduced number of SOX2⁺ cells and an increased number of Tuj1⁺, GFAP⁺ and Galc⁺ cells were observed in the non-initiating tumor cell population compared with GICs (Fig. 2A). There were significant differences in the expression levels of the stemness marker SOX2 ($P<0.001$) and differentiation markers GFAP ($P<0.001$), Galc ($P<0.001$) and Tuj1 ($P=0.002$) between GICs and matched non-initiating tumor cells (Fig. 2B).

Migration and invasion were greater in vitro for CD133-/A2B5⁺ GICs compared with matched non-initiating tumor cells. CD133-/A2B5⁺ GICs and matched non-initiating tumor cells were assessed for their invasive potential by a matrigel invasion assay and migratory capability by polycarbonate filters. The results revealed that an increase in invasive cells through the matrigel and more migratory cells through filters were observed in GICs comparing with matched non-initiating tumor cell populations. A total number of 72.7 ± 6.7 GICs in a field passed the matrigel, compared with 30.3 ± 4.6 non-initiating tumor cells (Fig. 3A). The number of migratory cells was 118.3 ± 8.5 in the GIC group when compared with 56.7 ± 5.7 in the non-initiating tumor cells group. The same events occurred in migratory assay (Fig. 3C). Quantified data demonstrated 2.42 folds more invasive (Fig. 3B) and 2.11 folds more migratory (Fig. 3D) cells in GICs comparing to matched non-initiating tumor cells populations. These findings indicated that the migratory and invasive potential of CD133-/A2B5⁺ GICs was greater than that of the matched non-initiating tumor cells

in vitro ($P<0.001$). No significant differences in migration or invasion were observed in GICs-RFP compared with GICs or non-initiating tumor cells-RFP ($P>0.05$). These results indicated that RFP transfection did not change the migration and invasion of GICs or matched non-initiating tumor cells, and cells with RFP may be used for *in vivo* invasive detection.

The expression levels of migration or invasion-associated markers were altered in CD133-/A2B5⁺ GICs. In addition to possessing the abilities of sphere-forming and growing, CD133-/A2B5⁺ GICs possessed certain genetic features during differentiation. Given that E-cadherin, ICAM-1, MMP-2, MMP-9 and TIMP3 are involved in the acquisition of highly migratory or invasive characteristics by different subsets of glioma cells, the mRNA and protein expression levels of these genes were examined using RT-qPCR and western blot analysis, respectively, to determine whether they were functionally associated with aggressive migration and invasion of GICs (Fig. 4A). The mRNA levels determined by RT-qPCR demonstrated that the expression levels of ICAM-1 ($P=0.016$), MMP-2 ($P<0.001$) and MMP-9 ($P<0.001$) were increased in CD133-/A2B5⁺ GICs compared with matched non-initiating tumor cells. The protein expression levels of ICAM-1, MMP-2 and MMP-9 were assessed using western blotting and matched the pattern of mRNA expression, demonstrating a significant increase in these proteins in GICs compared with matched non-initiating tumor cells (Fig. 4B). The mRNA and protein expression levels of E-cadherin and TIMP3 were also detected in GICs. A significant reduction in the mRNA ($P=0.003$) and protein expression levels of TIMP3 was observed in GICs compared with matched non-initiating

tumor cells. On the other hand, no significant difference in E-cadherin mRNA ($P=0.137$) or protein levels was observed between GICs and matched non-initiating tumor cells.

CD133⁺/A2B5⁺ GICs display greater invasive capacity compared with non-initiating tumor cells in vivo. Aggressive invasion of tumor cells into brain tissue is one of the most malignant characteristics of GBM. To test the infiltrative potential of GICs *in vivo*, CD133⁺/A2B5⁺ GICs or matched non-initiating tumor cells were transplanted into brains of athymic/nude mice through intracranial injection. Macroscopically, yellow-gray tumors were observed in mice with intracranial GICs or non-initiating tumor cells inoculation. Microscopically, the majority of tumor cells were primitive round or oval, distributed evenly and densely, after transplantation with GICs or non-initiating tumor cells. As presented in Fig. 5, in the brains implanted with GICs, aggressive invasion of tumor cells infiltrating into surrounding normal tissue was observed in 7 mice, and these tumor cells spread particularly far away from the original injection site. Obvious invasion occurred in 87.5% mice when implanted with CD133⁺/A2B5⁺ GICs. However, in the brains implanted with non-initiating tumor cells, the majority of tumor cells remained close to the injection site and no dispersal of tumor cells was detected following cell transplantation in all 8 mice. These data demonstrated that CD133⁺/A2B5⁺ GICs demonstrated infiltrating growth patterns and displayed greater invasive potential compared with matched non-initiating tumor cells.

Discussion

Surface markers of cancer stem cells such as CD133, CD15, A2B5, CD44 and $\alpha 6$ -integrin have been observed on distinct human glioblastoma cell populations (14-16). The phenotype of cancer stem cells varies substantially between patients, and tumors may contain multiple cancer stem cells, which are phenotypically or genetically distinct (17). Human gliomas consistently express A2B5 marker in a large percentage of cells, but CD133 expression is less abundant and less consistent, and the CD133⁺ population is almost entirely contained within the A2B5⁺ population (18). Human gliomas are composed of multiple populations of cells which have the capacity to form tumors. Notably, Ogden *et al* (18) identified a specific population of tumorigenic A2B5⁺ cells that were phenotypically distinct from CD133⁺ cells.

Tchoghandjian *et al* (19) reported that A2B5⁺ cells isolated from human GBM displayed neurosphere-like, self-renewal, asymmetrical cell division properties and had multipotent differentiation capability. As few as 1,000 cells derived from A2B5⁺ secondary spheres produced a tumor, and A2B5⁺/CD133⁺ and A2B5⁺/CD133⁻ cell fractions displayed high proliferative and migratory properties. For comparison with non-initiating tumor cells, the authors used the differentiated progeny of the CD133⁺/A2B5⁺ GICs, which provided an isogenic model system for detecting the influence of the GICs on migration and invasion. In the present study, the Transwell experiments indicated that GICs had greater migratory and invasive potential than matched non-initiating tumor cells *in vitro*.

Cell adhesion molecules, MMPs and TIMPs serve crucial roles in migration, invasion and metastasis and regulate

signaling pathways that control cell growth, survival, invasion, inflammation and angiogenesis (20-23). In the present study, higher expression levels of ICAM-1, MMP-2 and MMP-9 and lower expression of TIMP3 were observed in A2B5⁺/CD133⁻ GICs compared with matched non-initiating tumor cells. These results indicated that the expression levels of ICAM-1, MMP-2, MMP-9 and TIMP3 may be important in the process of aggressive GICs invasion. Expression changes in genes associated with migration and invasion in GICs compared with matched non-initiating tumor cells indicated that there may be multiple molecules and processes involved in stimulating GICs invasion *in vivo*.

Identification of individual or even small numbers of invasive tumor cells in the brain adjacent to tumor is difficult using standard immunohistochemistry or routine H&E techniques. RFP has been widely applied in the biomedical field as a non-enzymatic reporter gene. The RFP gene may be stably transduced into cancer cell lines, which subsequently express RFP at high levels *in vitro* and *in vivo*, including primary and metastatic tumor deposits. The method of RFP transfection is superior to H&E staining for the detection and study of physiologically relevant patterns of brain tumor invasion *in vivo* (24). Zhang *et al* (25) transferred DsRed2, an RFP gene, into malignant glioma cells. DsRed2 fluorescence clearly demarcated the primary tumor margins and readily allowed for the visualization of local invasion at the single-cell level in the brain adjacent to tumor. In the present study, in order to detect brain tumor invasion in an orthotopic glioma model of nude mice by fluorescence microscopy, a Lentivirus carrying the RFP gene was transfected into CD133⁺/A2B5⁺ GICs and matched non-initiating tumor cells. Infiltrating growth patterns and aggressive invasion potential were demonstrated in xenografts from CD133⁺/A2B5⁺ GICs compared with matched non-initiating tumor cells. The present results were in accordance with previous studies, which also demonstrated that glioblastoma CD133⁺ cells exhibited properties of stem cells, and generated more aggressive tumors than CD133⁻ cells when engrafted intracerebrally into nude mice (26-28).

In conclusion, the present study demonstrated that these CD133⁺/A2B5⁺ cells fulfilled the criteria of brain tumor initiating cells *in vitro*. These cells displayed significant migratory and invasive properties. These infiltrated cells in the invasive fronts may be responsible for rapid tumor recurrence after conventional treatments. Current hypotheses relating to cancer stem cells generally propose targeting CD133⁺ cells to inhibit GBM recurrence, but the present study indicated that CD133⁺/A2B5⁺ GICs are also an important cell subpopulation with great invasive potential that should not be ignored. Targeting both CD133⁺ and CD133⁻ invasive GICs may effectively reduce GBM invasion and recurrence.

Acknowledgements

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