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Proliferative status of tumor stem cells may be correlated with malignancy grade of human astrocytomas

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1. ABSTRACT

Tumor stem cells are implicated in tumor initiation and maintenance. Recent studies have shown that a subpopulation of cells isolated from brain tumors can form neurospheres *in vitro*, and have multiple characteristic properties observed in neural stem cells. *In vivo* implantation of these cells can induce tumors that phenocopy original tumors, suggesting that tumor stem cells are involved in brain carcinogenesis. We found that a population of cells in human glioblastoma multiforme expressed multiple protein markers of neural stem cells including nestin, TUC-4, doublecortin and beta III-tubulin. In contrast, these markers were not expressed in human capillary hemangioblastoma or meningioma. Double immunolabeling showed that a portion of doublecortin-, beta III-tubulin-, TUC-4- and nestin-positive cells express Ki67 antigen, a cell proliferation marker. To investigate further whether these properties of tumor stem cells are correlated with their biological behavior, immunohistochemistry was performed on brain sections from astrocytomas of different grades using antibodies against neural stem cell markers. The number of cells expressing Ki67 antigen and neural stem cell markers was increased in relation to worsening histological grade of astrocytomas, indicating that the capacity for tumor stem cell proliferation may be clinically relevant. Thus, tumor stem cells in astrocytomas may be involved in carcinogenesis.

2. INTRODUCTION

Brain tumors comprise heterogeneous cell types ranging from immature and highly proliferative to lineage-committed, differentiated cells. Little is known about the mechanisms underlying brain tumor initiation and progression, although major advances have been made in understanding the molecular genetic alterations in some brain tumors (1, 2). Current evidence suggests that a small fraction of tumor cells — tumor stem cells — may be responsible for the initiation and maintenance of tumors (1). Tumor stem cells were first demonstrated in leukemia and multiple myeloma, in which only a small subset of cells (1-4%) form spleen colonies *in vitro* (3, 4). These cells can be isolated and transplanted into non-obese diabetic mice with severe combined immunodeficiency disease (NOD/SCID mice) to form tumors that phenotypically resemble the patient's original tumor (5-7). Similar findings have been observed in breast cancer, where cells are phenotypically heterogeneous, and only a small percentage are clonogenic in culture *in vitro* (8) and form tumors in mice (9).

Recent studies show that a minor fraction of cells from brain tumors express the neural stem cell surface marker CD133, and that these $CD133^+$ cells can form tumor-derived neurospheres containing cells with many characteristics of neural stem cells *in vitro* (10-14). Moreover, injection of as few as 100 $CD133^+$ cells from human glioblastomas or medulloblatomas into NOD/SCID mice produced tumors that matched the patient's original tumor phenotype, whereas grafts resulting from injection of 10^5 $CD133^-$ cells did not yield tumors (12, 15). This suggests that most brain tumor cells are unable to proliferate extensively, and that only a small subset of ($CD133^+$) cells are consistently clonogenic, and might represent brain tumor-initiating cells.

In addition to the behavioral similarity between brain tumors and neural stem cells *in vitro* and *in vivo*, many brain tumors contain cells that express neural stem cell markers, such as the intermediate filament protein nestin (11, 16), the polysialylated (embryonic) form of nerve cell adhesion molecule (PSA-NCAM) (17), and the RNA-binding protein Musashi. Similarities also exist between the mechanisms that regulate self-renewal of neural stem cells and brain tumor cells (18). For example, the Wnt signaling cascade has been implicated in self-renewal of hematopoietic stem cells and neural stem cells, as well as in the growth of brain tumor cells (19).

Although these findings are consistent with the view that brain tumors may be derived from stem cells, the relationship of these cells to the clinical behavior of brain tumors is uncertain. Thus far, no specific marker of brain tumor aggressiveness has been found (20), and traditional histopathology yields only limited information about tumor behavior (2). In this study, 32 human primary astrocytic tumors were examined by immunohistochemistry for multiple neural stem cell markers. We found that a subpopulation of tumor cells express neural stem cell markers together with the cell-proliferation marker Ki67 antigen. Furthermore, the number of tumor stem cells present in tumors increased with increasing grade of the astrocytoma, suggesting that the proliferation of tumor stem cells correlates with the grade of malignancy. This observation could have implications not only for understanding brain tumorigenesis, but also for the clinical diagnosis, prognosis, and treatment of brain tumors.

3. MATERIALS AND METHODS

3.1. Brain tumor tissue specimens

Human astrocytic tumor specimens were obtained from patients undergoing surgical resection at the Department of Neurosurgery, Huashan Hospital, Fudan University (Shanghai, China). Tumors were classified by the attending neuropathologist at the Department of Pathology of Fudan University according to World Health Organization (WHO) guidelines (21). Patients ranged from 4 to 75 years of age, with a median age of 36 years. Of the 32 astrocytic tumor tissues, 9 samples were classified as pilocytic astrocytomas (non-invasive, WHO grade I), 9 as astrocytomas (WHO grade II), 5 as anaplastic astrocytomas (WHO grade III), and 9 as glioblastma multiforme (WHO grade IV). Additional details are given in Table 1. The protocol for this study was approved by the Institutional Review Board. Tissue was fixed in 10% formaldehyde and embedded in paraffin for histological examination.

3.2. Immunohistochemistry

Human brain tumor specimens embedded in paraffin were cut in 6-μm sections, which were deparaffinized with xylene and rehydrated with ethanol, following antigen retrieval with antigen unmasking solution (Vector) according to the manufacturer's instructions. Peroxidase activity was blocked with 1% H_2O_2 and sections were incubated in blocking buffer (2% horse serum, 0.2% Triton X-100, 0.1% BSA in PBS) for 1 hr at room temperature. Immunohistochemistry was performed as previously described (22). Primary antibodies used were: (*i*) mouse monoclonal anti-PSA-NCAM (Chemicon; 1:250), (*ii*) affinity-purified rabbit anti-Musashi (Chemicon; 1:500), (*iii*) affinity-purified goat polyclonal anti-doublecortin (DCX) (Santa Cruz Biotechnology; 1:200), (*iv*) mouse monoclonal anti-beta III-tubulin (TUJ-1; Covance; 1:250), (*v*) rabbit polyclonal anti-TUC-4 (Chemicon; 1:1,000), (*vi*) affinity-purified goat polyclonal anti-NeuroD (Santa Cruz Biotechnology; 1:200), (*vii*) mouse monoclonal anti-human specific nestin (Chemicon; 1:200), and (*viii*) mouse monoclonal anti-human Ki67 antigen, clone MIB-1 (Zymed Laboratories; 1:50). Sections were washed with 0.1% Tween 20 in PBS, incubated at room temperature for 60 min with horseradish

Table 1. Expression of neural stem cell proteins in human astrocytic tumors

Tumor	Sex	Age	Site of tumor	DCX	TUC-4	TUJ-1	Musashi	Nestin[1]
Grade I								
G151	M	6	cerebellum	±	++++	+	+++	+
G182	F	4	v.cerebelli	+	++++	+++	++++	++
G196	F	18	temporal lobe	++	++	++	+++	+
G207	F	14	left cerebellum	ND	+++	ND	++	±
G240	M	13	cerebellum	-	++	++	+++	+
Grade II								
G255	F	58	frontal lobe	+++	+++	+	+++	++
G262	M	37	frontal lobe	+	++++	+++	++++	+++
G265	M	16	frontal lobe	++	+	++++	++	+++
G282	M	28	frontal lobe	+	++	++++	+++	+++
G297	M	46	frontal lobe	++	++	+	+++	++
Grade III								
G188	F	39	parietal lobe	++	++	+	+++	+++
G232	M	31	parietal lobe	+	+	+++	+++	++
G285	F	70	temporal lobe	++	++	++++	++	+
H168	M	52	temporal lobe	ND	ND	++	++++	+++
Grade IV								
G259	F	44	temporal lobe	+++	+++	++++	++++	+++
G271	M	50	occipital lobe	+++	++	+++	++++	++++
G277	M	60	temporal lobe	+++	+++	+++	+++	+++
G286	F	14	temporal lobe	++	+	++	+++	++++
G294	M	5	parietal lobe	+	++	++	++	++
H2982	M	67	temporal lobe	++++	++++	+	++++	++++

Immunoreactivity of neural stem/progenitor protein markers in human primary astrocytic tumors is given as: ND, not determined; -, no signal; ±, minor signal; +, weak signal; ++, moderate signal; +++ strong signal; ++++ very strong signal. [1] Kruskal-Wallis statistic, $H = 11.505$, $p = 0.012$

peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology; 1:3,000), and washed three times for 15 min with PBS. The horseradish peroxidase reaction was detected with diaminobenzidine and H_2O_2. Alternating sections were incubated without primary antibody as a control.

3.3. Double-label immunohistochemistry

Double-label immunohistochemistry of brain tumor sections to detect coexpression of Ki67 antigen with early neuronal marker proteins was performed as previously described (22, 23), using the primary antibodies listed above. The secondary antibodies were FITC-conjugated rat-absorbed donkey anti-goat or anti-mouse IgG and rhodamine-conjugated donkey anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch; 1:200). Fluorescence signals were detected with a Nikon E800 microscope at excitation/emission wavelengths of 535/565 nm (rhodamine, red) and 470/505 (FITC, green). Results were recorded with a Magnifire digital camera (ChipCoolers, Warwick, RI). Controls included omitting or preabsorbing primary antibody or omitting secondary antibody. Selected images were viewed at high magnification using a Nikon PCM-2000 confocal laser-scanning microscope, and 3-dimensional images were reconstructed using Imars software to confirm colocalization of markers.

3.4. Cell counting

Immunopositive cells were counted in two to eight, 400 × fields per section, chosen from the area of maximal labeling, using an eyepiece grid covering an area of 0.0625 mm^2. Vessels and blood cells were excluded from analysis. The Ki67 labeling index was defined as the number of $Ki67^+$ cells, expressed as a percentage of the total number of cells in the evaluated area.

3.5 Data analysis

Data were expressed as mean ± SEM from at least three experiments. Except where indicated otherwise, ANOVA and Student's *t* test were used for statistical analysis, with $p < 0.05$ considered significant.

4. RESULTS

To determine if our astrocytomas contained a subpopulation of tumor cells with features of neural stem cells, we first performed immunohistochemistry on glioblastomas (WHO grade IV astrocytic tumor) from 9 patients, using antibodies against the following proteins: (1) DCX, a tubulin-binding protein involved in neuronal migration (24, 25); (2) PSA-NCAM, a highly polysialylated embryonic form of neural cell adhesion molecule (26); (3) nestin, an intermediate filament protein expressed by neural stem cells; (4) beta III-tubulin, an earlier marker for neuronal cells *in*

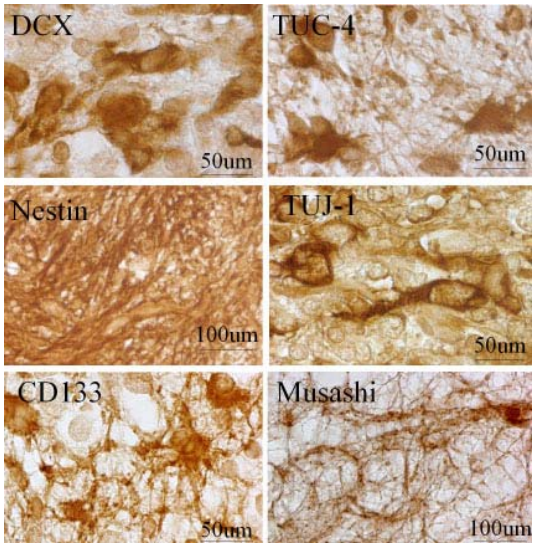

Figure 1. Expression of neural stem cell proteins in human grade IV astrocytoma (glioblastoma). Paraffin-embedded sections of human glioblastomas were immunostained with antibodies against the neural stem cell proteins DCX, beta III-tubulin (TUJ-1), TUC-4, nestin, CD133 and Musashi, each of which was expressed in a subpopulation of tumor cells.

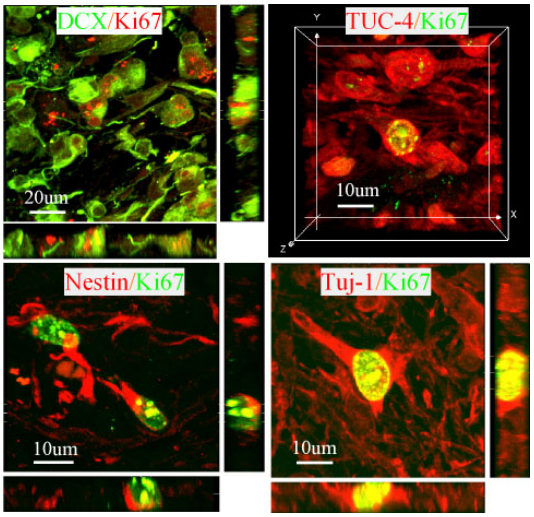

Figure 2. Proliferation state of neural stem cell protein-immunoreactive tumor cells. Double-immunolabeling was performed using anti-Ki67 antigen and antibodies against neural stem cell proteins. Z-stack images were recorded using a confocal laser-scanning microscope and images were reconstructed to confirm that putative neural stem cells expressed the cell-proliferation marker protein, Ki67 antigen. Note that Ki67 antigen is localized primarily to nuclei, whereas the other markers are primarily extranuclear.

vivo (27); (5) CD133, a novel five-transmembrane segment cell-surface protein originally shown to be a hematopoietic stem cell marker and recently found to be a marker of normal human neural stem cells (28); (6) Musashi, a RNA-binding protein expressed in stem cells in the adult subventricular zone (SVZ) (29), and (7) TUC-4, a protein expressed early in neuronal differentiation in rat (30). As shown in Figure 1, DCX, nestin, TUC-4, beta III-tubulin, CD133, Musashi and PSA-NCAM were each expressed in glioblastomas. In addition, multiple neural stem cell markers were typically expressed in the same cell by analysis of double immunostaining. In contrast, these marker proteins were not expressed in the human capillary hemangioblastoma or meningioma, except for nestin, which present tumor blood vessels (not shown).

We then asked whether tumor cells immunoreactive for early markers of neuronal lineage exhibited features associated with cell proliferation. Double immunolabeling was conducted using antibodies against Ki67 antigen, which binds to nuclear proteins in the G1, S, G2 and M phases of the cell cycle (31), together with antibodies against the neural stem cell markers listed above. Immunopositive cells were scanned by confocal laser scanning microscopy and z-stack images were reconstructed using Imars software to ensure that both proteins were truly expressed in the same cell. As shown in Figure 2, a subset of cells that expressed neural stem cell markers was also reactive for Ki67 antigen, consistent with a proliferative phenotype. Our results further documented that brain tumor stem cells might be present in human primary astrocytic tumors.

Next, we examined the relationship between malignancy grade and expression profiles of several neural stem cell markers in these tumors by immunohistochemistry. As summarized in Table 1, most neural stem cell markers were unlikely tie to the histological grade of brain tumors. The observation was further confirmed by Western blots (data not shown). However, there was a statistically significant association between nestin expression and malignancy grade, and a lesser (but not significant; $H = 6.977$, $p = 0.095$) tendency towards increased DCX expression in higher-grade tumors. We then asked whether proliferating tumor stem cells might be implicated in the clinical behavior of astrocytomas. We first examined the relationship between malignancy grade and the Ki67 labeling index (Figure 3). Ki67 labeling was increased in high- compared to low-grade human astrocytomas. Double immunolabeling indicated that these Ki67-positive cells expressed at least one or several neural stem cell markers, suggesting these Ki67-positive cells were proliferating tumor stem cells. When the number of cells that co-expressed Ki67 antigen and nestin or Ki67 antigen and DCX was counted, both Ki67 antigen+/nestin+ and Ki67+/DCX+ cells were significantly increased in high- (III-IV) compared to low- (I-II) grade astrocytomas (Figure 4). Our findings might have the predicted value for prognosis and provide additional guidelines for the treatment of brain tumors

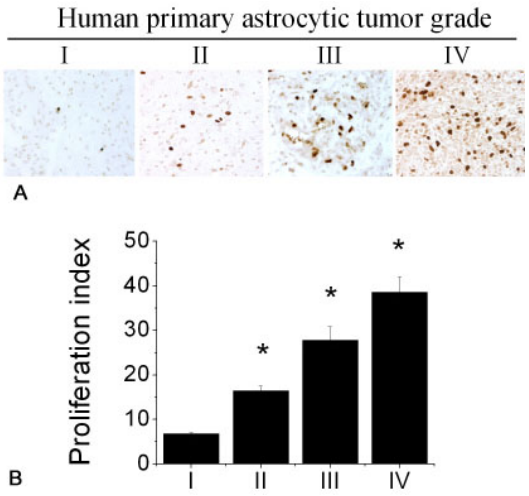

Figure 3. Proliferation index of human astrocytic tumors in relation to histological grade. (**a**) Sections from astrocytomas of different grades were immunostained with anti-Ki67 antigen. (**b**) Proliferation index of astrocytomas of different grades was calculated as the percentage of all tumor cells that were Ki67-immunopositive. Data are mean values ± SEM. *, $p < 0.05$ compared to grade I (ANOVA and *post hoc t*-test).

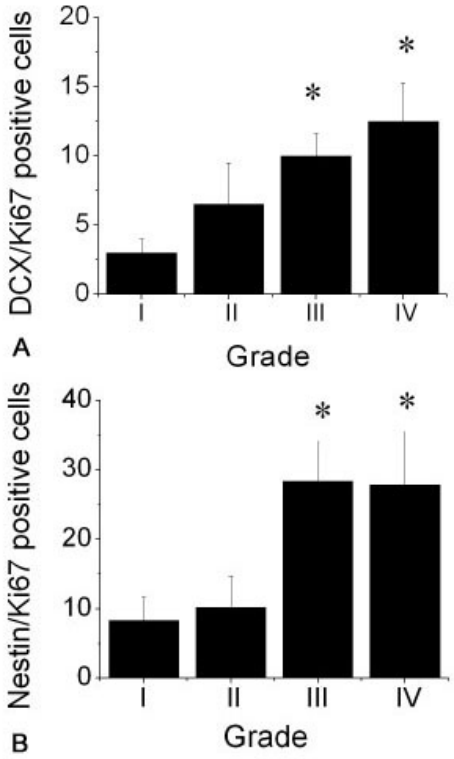

Figure 4. Percentage of all tumor cells from human astrocytomas of different histological grades that co-expressed Ki67 antigen and (**a**) DCX or (**b**) nestin, as analyzed by confocal laser scanning microscopy. Data are mean values ± SEM. *, $p < 0.05$ compared to grade I (ANOVA and *post hoc t*-test).

5. DISCUSSION

The major finding of this study is that human astrocytomas express markers associated with cell proliferation and with the neural stem cell phenotype, and that the number of tumor cells that exhibit certain of these markers correlates with histological malignancy grade. Several prior studies have shown that brain tumor stem cells capable of proliferating *in vitro* or *in vivo* are present in human astrocytomas. However, their biological significance is not fully understood. The relationship between proliferative tumor stem cell number and malignancy that we observed suggests a functional connection between tumor stem cells and the clinical behavior of human gliomas.

We observed a correlation between malignancy grade and the expression of three markers – Ki67 antigen, nestin and DCX. Ki67 antigen is a cell-cycle protein that is lineage-nonspecific, whereas nestin is expressed in neural stem cells that give rise to either neurons or glia. DCX, however, is associated specifically with neuronal differentiation, and is charcteristically found in migrating neurons. The expression of DCX in astrocytic tumors, and the association between $Ki67^+/DCX^+$ cells and tumor malignancy, is therefore somewhat surprising.

Whether gliomas arise through the dedifferentiation of mature glia or from tissue stem cells that remain sequestered in the brain throughout life is uncertain (35). Some prior studies suggest that tumor stem cells express markers of glial, but not neuronal, lineage *in vivo*, and that neuronal markers are observed only as an artifact of cell culture (10). In other cases, neuronal markers (e.g., beta III-tubulin) have also been found *in vivo* (12). We studied a more extensive panel of neural stem cell protein markers, and found that the intermediate filament protein nestin, which characterizes neuroepithelial stem cells, and the immature neuronal protein DCX, which is expressed in migrating neurons, were both present in tumor stem cells, as defined by immunoreactivity for one or more neural stem cell markers together with the cell-cycle protein Ki67 antigen. The occurrence of neuronal lineage markers like DCX in tumor-associated stem cells may be more consistent with aberrant proliferation of tissue stem cells (which might give rise to cells with features of both glial and neuronal lineage) than with dedifferentiation of mature astroglia (which might be expected to recapitulate only glial attributes).

Two prior studies that used gene expression profiling to characterize the relationship between marker expression in astrocytomas and survival yielded results that are, in some sense, in conflict with our findings. In one study (32), a group of genes described as "neurogenesis genes" was included in a hierarchical expression cluster found to be predictive of prolonged survival from grade III-IV astrocytomas.

The genes in question were bone morphogenetic protein 2, delta-like 3, histone deacetylase 4, endothelin receptor type B, hairy/enhancer-of-split related, and neurotrophic tyrosine kinase receptor type 2, none of which we studied. Ki67 antigen, protein expression of which we found to correlate with malignancy grade, clustered with other proteins whose expression was linked to poor prognosis. In another study (33), DCX was one of three genes (the others being osteonectin and semaphorin 3B) whose expression correlated with prolonged survival from grade IV astrocytomas in patients >50 years of age. Explanations for the differences between these findings (suggesting that neurogenesis marker expression is prognostically favorable) and ours (suggesting the opposite) include differences in the range of histological tumor grades studied, the nature of the markers (RNA vs. protein) examined, the manner in which marker expression was quantified (amount of marker expression vs. number of marker-expressing cells), and the biological endpoints used (survival, which also reflects response to treatment and other clinical factors, vs. histological grade). In addition, in all these studies, the endpoints were most strongly associated with combinations of, rather than individual, markers, such as expression of Ki67 antigen together with nestin or DCX, or clusters of genes.

Previous studies show that the proliferation rate of tumor cells has a strong association with outcome from childhood malignant gliomas (34-37). Our results are consistent with this notion. However, we noted that the expression level of different tumor stem cell proteins in astrocytomas varied, even within the same histological classification. One explanation for this finding may be that the sequential expression of neural stem cell markers that is observed in normal neuroglial development is not preserved during tumorigenesis. Another possibility is that not all the tumor stem cells in our brain tumor specimens originated from the tumor. A variety of brain lesions stimulate the proliferation and recruitment of neural stem cells from distant sites to the vicinity of the lesion. For example, transplanted neural stem cells migrate preferentially to the tissue surrounding rat brain gliomas, even when transplants and tumor are placed in opposite cerebral hemispheres (38). Neural stem cells might also arise in peritumoral brain tissue in response to the tumor, as a similar phenomenon has been suggested to occur in the ischemic penumbra surrounding some brain infarcts (39). Consequently, some neural stem cells found in specimens dissected from patients with brain tumors may have arisen in normal brain in response to the tumor, and express markers that reflect a range of maturational stages, unrelated to expression patterns found in the inciting tumor. Some markers, like nestin, are also associated with tumor vasculature.

The clinical significance of our findings, i.e., the extent to which Ki67 antigen, nestin or DCX protein expression may be relevant to prognostication or predictive of response to therapy in astrocytoma, is uncertain. However, they raise certains issues. For example, if the proliferative state of tumor stem cells influences clinical tumor behavior, measures to stimulate neuroproliferation for neuronal replacement in neurodegenerative diseases might also enhance the growth of occult brain tumors. Conversely, tumor stem cells might represent a novel target for cancer therapy. In this regard, non-tumor tem cells have been shown to be sensitive to antimitotic drugs (40) and radiation (41).

Finally, if astrocytomas do arise from neural stem cells, where do these cells originate? Neurogenesis is thought to occur primarily in the dentate gyrus and subventricular zone of the adult brain, whereas astrocytomas most commonly arise in white matter of the cerebral hemispheres, brainstem or cerebellum, or in the optic nerves. It is, therefore, of interest that neural stem cells with the capacity to differentiate along both glial and neuronal lineages have been identified in subcortical white matter of both human (42) and rodent (20) brain.

6. ACKNOWLEDGEMENT

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Abbreviations: NOD/SCID mice: non-obese diabetic mice with severe combined immunodeficiency disease, SVZ: subventricular zone, PSA-NCAM: polysialylated form of nerve cell adhesion molecule, WHO: World Health Organization, DCX: doublecortin

Key Words: Tumor, Cancer, Neoplasia, Stem Cell, Neurogenesis, Astrocytoma, Glioblastoma, Glioma, Glial Cell, Brain

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