Expansion of CD133-positive glioma cells in recurrent de novo glioblastomas after radiotherapy and chemotherapy

Laboratory investigation

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Object. Recent evidence suggests that a glioma stem cell subpopulation may determine the biological behavior of tumors, including resistance to therapy. To investigate this hypothesis, the authors examined varying grades of gliomas for stem cell marker expressions and histopathological changes between primary and recurrent tumors.

Methods. Tumor samples were collected during surgery from 70 patients with varying grades of gliomas (Grade II in 12 patients, Grade III in 16, and Grade IV in 42) prior to any adjuvant treatment. The samples were subjected to immunohistochemistry for MIB-1, factor VIII, GFAP, and stem cell markers (CD133 and nestin). Histopathological changes were compared between primary and recurrent tumors in 31 patients after radiation treatment and chemotherapy, including high-dose irradiation with additional stereotactic radiosurgery.

Results. CD133 expression on glioma cells was confined to de novo glioblastomas but was not observed in lower-grade gliomas. In de novo glioblastomas, the mean percentage of CD133-positive glioma cells in sections obtained at recurrence was 12.2% ± 10.3%, which was significantly higher than that obtained at the primary surgery (1.08% ± 1.78%). CD133 and Ki 67 dual-positive glioma cells were significantly increased in recurrent de novo glioblastomas as compared with those in primary tumors (14.5% ± 6.67% vs 2.16% ± 2.60%, respectively). In contrast, secondary glioblastomas rarely expressed CD133 antigen even after malignant progression following radiotherapy and chemotherapy.

Conclusions. The authors’ results indicate that CD133-positive glioma stem cells could survive, change to a proliferative cancer stem cell phenotype, and cause recurrence in cases with de novo glioblastomas after radiotherapy and chemotherapy.

Key Words • cancer stem cell • CD133 • treatment resistance • de novo glioblastoma • immunohistochemistry • oncology

MALIGNANT gliomas, especially glioblastomas, are among the most lethal primary human malignancies, showing rapid growth, high invasiveness and vascularity, and occasional dissemination into the CSF space.15 Although temozolomide plus radiation, a common treatment for glioblastoma, has demonstrated a significantly increased survival, the prognosis of patients with glioblastoma still remains poor and the median survival time is only 14.6 months.30

Cancer stem cells are rare tumor cells characterized by their ability to induce tumorigenesis and to self-renew. Cancer stem cells may also determine the biological behavior of tumors, including responses to therapy.12,24 CD133 has been suggested to be a cancer stem cell marker in malignant brain tumors because only CD133-positive cells from brain tumor biopsy material were able to initiate brain cancer in a mouse model.27,28 Growing evidence indicates CD133 expression to be related to a poor prognosis, tumor aggressiveness, and treatment failure. Bao et al.1 reported that a glioma stem cell subpopulation expressing CD133 contributes to radioresistance in freshly isolated cultured cells and in a rodent model of glioblastoma via preferential activation of the DNA damage response. Studies of cultured cells and experimental animal models support this hypothesis.5,10,13,19 However, the validity of CD133 as a glioma stem cell marker and its clinical ramifications are still controversial.4 The identification of

Abbreviations used in this paper: EBRT = external-beam radiation therapy; GKS = Gamma Knife surgery.
CD133-negative glioblastoma-derived cancer stem cells has raised the question of whether CD133 serves as a universal enrichment marker for glioma stem cells.\textsuperscript{3,8,20,29} Another controversial issue includes the lack of reliable data in the detection of CD133 antigen by immunohistochemistry.\textsuperscript{11}

We recently reported preliminary data indicating that CD133-positive glioma stem cell accumulation occurs after high-dose irradiation by Gamma Knife surgery (GKS) plus external-beam radiation therapy (EBRT) in malignant gliomas.\textsuperscript{31} We used the primary antibody, a C24B9 clone, recognizing a nonglycosylated extracellular epitope of CD133 and applied a biotin-free highly sensitive indirect immunohistochemical method to detect CD133 antigen. Using this immunodetection system, we successfully demonstrated specific signals for CD133 antigen on paraffin sections of malignant gliomas. In our present study, to further test the validity of CD133 as a glioma stem cell marker and its clinical utility, we examined gliomas of varying grades for their stem cell marker expressions and compared the expression of CD133 positive glioma stem cells in histological sections from primary and recurrent gliomas after radiation and chemotherapy.

**Methods**

**Patients and Tissue Collection**

Histopathological studies were performed on 102 tumor tissue samples obtained from 70 patients with varying grades of gliomas who underwent resection from 2001 to 2008. We studied 70 samples from primary surgery, 29 from the surgery at recurrence, and 3 from autopsies. Tumor tissues were fixed in 4% neutral-buffered formalin and embedded in paraffin. Tissue sections were stained with H & E and were histologically classified according to the WHO classification. Tumor tissues from the primary surgery were obtained from resection prior to radiotherapy and chemotherapy. After surgical removal, EBRT was administered using a LINAC with 2 Gy as the daily fraction and 54 Gy as the median total dose. From 2001 to 2005, patients with high-grade gliomas received adjuvant chemotherapy consisting of 4 or 5 cycles of intravenous cisplatin and etoposide or a combination of ACNU and vincristine. Thereafter, temozolomide was given as an adjuvant chemotherapy or concomitant chemoradiotherapy. From 2001 to 2006, patients underwent GKS after initial surgical removal in addition to EBRT. The median tumor volume treated at the time of GKS was 18.2 cm\(^3\), and the median GKS dose was 18 Gy (range 12–25 Gy) with the median prescription isodose line being 50% (range 40%–60%). Contrast-enhanced MR images were obtained serially in each patient to monitor tumor progression. Repeat surgical removal was considered for tumor recurrence after the discovery of progressive changes on MR images and/or t-[methyl-\(^{13}\)C]methionine PET images, regardless of the presence of progression of neurological deficits. Complete follow-up was obtained for all patients. Informed consent was obtained from patients and/or their guardians, and the study was approved by the ethics committee of the Tokyo Medical and Dental University.

**Immunohistochemistry**

Immunohistochemical detection was performed using the following antibodies: rabbit antibody against CD133 (C24B9, Cell Signaling), mouse antibody against Ki 67 antigen (MIB-1, Dako), rabbit antibodies against factor VIII–related antigen (Dako), nestin (AB5922, Chemicon), and GFAP (Dako). Sections were pretreated in an autoclave to enhance immunoreactivity.\textsuperscript{33} The catalyzed signal amplification method with a CSAII kit (Dako) was used for the detection of CD133 antigen, and the labeled streptavidin-biotin method with an LSAB kit (Dako) was used for other antigens. Sections were developed with diaminobenzidine (Dako) or Vector VIP substrate (Vector Laboratories) and counterstained with hematoxylin. Ki 67 staining indices were determined by counting more than 500 nuclei in 3 or more randomly selected high power fields (400x magnification). Tumor blood vessel density was determined by counting the number of blood vessels per area in sections stained with antibodies against factor VIII-related antigens. The percentage of CD133-positive cells per tumor area in histological sections was calculated according to a previously reported method.\textsuperscript{31} Because of the variation in CD133-positive cell distributions in histological sections, we selected viable tumor tissues, excluding necrosis and nontumorous brain tissues, and determined the percentage of CD133-positive cells in several small sections of a whole histological section. The percentage of CD133-positive cells in tumor tissues in a whole section was calculated by counting more than 500 cells in each small section and by measuring actual areas with ImageJ software for Macintosh. Immunohistochemically stained slides were reviewed independently by 3 investigators who were unaware of the clinical data. The mean of the data obtained from these investigators was used for the analysis.

**Statistical Analysis**

A JMP program (version 5, SAS Institute Inc.) was used for statistical analysis. Parametric data were expressed as means ± SD. The differences in mean values were compared by the unpaired t-test. Paired data before and after treatment were compared by the paired t-test. For all statistical analyses, p < 0.05 was considered significant.

**Results**

**Identification of CD133-Positive Glioma Cells on Histological Sections From Patients With Varying Grades of Gliomas**

We examined the CD133 expressions on surgical sections from 70 patients with varying grades of gliomas prior to adjuvant treatment. The patient group contained 12 Grade II diffuse astrocytomas, 13 Grade III anaplastic astrocytomas, 3 Grade III anaplastic oligodendrogliomas, and 42 Grade IV glioblastomas. Using the CSAII detection system and rabbit monoclonal CD133 antibody—a C24B9 clone—as a primary antibody, positive immunoreactivity for CD133 antigen was clearly identified on the cell membranes but not in the nuclei of glioma cells (Fig.
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1A, C, and D). No reactivity was observed when a nonspecific isotope control instead of the primary antibody was used as a negative control (Fig. 1B). No CD133-positive glioma cells were detected in the Grade II and Grade III gliomas. CD133-positive glioma cells were found in 28 of the 42 patients with Grade IV glioblastomas. When measuring the percentage of CD133-positive cells per tumor area in histological sections from primary surgery, CD133 was expressed on no more than 1% of cells in 17 glioblastomas, while higher percentages (1%–13.7%) of CD133-positive cells were found in 11 other glioblastomas (Fig. 1E). CD133-positive glioma cells usually appeared in clusters and were often located close to tumor blood vessels (Fig. 1C) and were occasionally seen in pseudo-palisade formations (Fig. 1D). Most of the CD133-positive glioma cells lacked Ki 67 immunopositivity as shown by double immunostaining with MIB-1 and CD133 antibodies (Fig. 1D).

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Thirty-one of the 70 varying-grade gliomas were examined histologically at the time of tumor recurrence or autopsy. Histological diagnoses at the primary surgeries of the 31 patients are shown in Table 1. Twenty-eight patients underwent a second surgery at recurrence after radiation treatment with or without chemotherapy, and 2 patients were examined at autopsy. Case 13 was examined at both recurrence and autopsy. At recurrence, all diffuse astrocytoma and anaplastic astrocytoma cases showed malignant progression and were diagnosed as secondary glioblastomas. The site of recurrence was local in all but 1 patient who had a remote-site recurrence. The 3 cases examined at autopsy showed both local and remote-site tumor recurrence.

We compared the expressions of stem cell markers, CD133 and nestin, between sections of primary and recurrent tumors by immunohistochemistry. We examined both primary and recurrent tumor sections in 20 patients with de novo glioblastomas. CD133-positive glioma cells were found in 14 of the primary and all of the recurrent tumors in these 20 de novo glioblastoma cases. CD133-negative glioma cells were infrequent in sections at primary surgery but were increased in sections obtained at recurrence in all the patients with de novo glioblastomas (Fig. 2A and B). Immunoreactivity for nestin was diffusely positive in all glioma cells in sections obtained at both primary surgery and recurrence and did not correspond to CD133 immunopositivity (data not shown). CD133-positive cells were positive for GFAP immunostaining. Two autopsy cases of de novo glioblastomas with multiple disseminations showed extensive distributions of CD133-positive glioma cells even in sections obtained from the remote-site recurrence (Fig. 2C–H). In de novo glioblastomas, the mean percentage of CD133-positive glioma cells in sections obtained at recurrence was 12.2% ± 10.3%, which was significantly higher than the percentage of CD133-positive tumor cells in sections from the primary surgery (1.08% ± 1.78%, p < 0.00007, paired t-test) (Fig. 3 upper). In contrast, none of the 4 secondary glioblastomas that had arisen from Grade II diffuse astrocytomas following radiotherapy expressed CD133 immunopositivity (Fig. 3 lower). CD133-positive glioma cells were observed in 2 of 4 secondary glioblastomas that had arisen from Grade III anaplastic astrocytomas following radiotherapy, but their frequency was very low. In anaplastic oligodendroglioma cases, CD133 immunoreactivity was not observed in sections from either primary surgeries or those at recurrence after radiotherapy with or without chemotherapy.

We compared the effect of radiation exposure on tumor blood vessels between EBRT alone and high-dose irradiation (EBRT plus GKS) in de novo glioblastomas with local failure. The number of factor VIII–positive tumor blood vessels in sections of recurrent tumors after high-dose irradiation was significantly smaller than that in sections of primary tumors (p < 0.005, paired t-test), while it was not significantly different between primary and recurrent tumors after EBRT alone (Fig. 4). The frequency of CD133-positive glioma cells was significantly increased in recurrent tumors of de novo glioblastomas both after EBRT alone and after EBRT plus GKS, although the increase in frequency after EBRT alone appeared smaller than that after EBRT plus GKS. Our results indicate that CD133-negative glioma stem cells can survive high-dose radiation, even though high-dose radiation can effectively induce damage in tumor blood vessels.

Proliferative Potentials of CD133-Positive Glioma Cells in Recurrent De Novo Glioblastomas After Radiotherapy and Chemotherapy

Since the frequency of CD133-positive glioma cells was significantly increased in recurrent de novo glioblastomas, we further examined the proliferative potentials in CD133-positive glioma cells by double immunostaining of CD133 and Ki 67 antigens. In sections from primary surgeries, CD133-positive glioma cells rarely showed Ki 67 immunoreactivity, whereas surrounding CD133-negative glioma cells exhibited high levels of Ki 67 staining (Figs. 1D and 5A). In contrast, in sections from glioblastomas recurring after radiotherapy and chemotherapy, Ki 67 immunoreactivity was frequently found in CD133-positive glioma cells (Fig. 5B). CD133-positive glioma cells were found in 14 of the primary and all of the recurrent tumors in de novo glioblastoma cases. The mean Ki 67 index of CD133-positive glioma cells in recurrent tumors (14.5% ± 6.67%, n = 20) was significantly higher than that in primary tumors (2.16% ± 2.60%, n = 14, p < 0.000001, unpaired t-test), while Ki 67 indices of CD133-negative glioma cells in whole sections were significantly lower in recurrent tumors than in primary tumors (p < 0.02; Fig. 5C). A comparison of 14 cases exhibiting CD133-positive cells before and after treatment revealed the Ki 67 indices of CD133-positive glioma cells to also be significantly higher in recurrent glioblastomas than in primary tumors (p < 0.0001, paired t-test).

Discussion

Human gliomas contain a small population of cells with stem cell features. In malignant brain tumors, CD133 has been suggested to be a cancer stem cell marker.
Fig. 1. Immunohistochemical analysis for CD133 in gliomas of different grades at primary surgery.  

A: CD133 immunohistochemistry (brown, DAB) on paraffin section from Case 6 (de novo glioblastoma). Positive staining is seen in clusters. Counterstained with hematoxylin; bar = 100 μm.  

B: Nonspecific isotype control staining on the serial section of that shown in panel A. Bar = 100 μm.  

C: CD133-positive glioma cells around the tumor vasculature designated by the arrowhead. Bar = 20 μm.  

D: Double immunostaining for CD133 (brown, DAB) and Ki 67 (purple, Vector VIP substrate) on a section from Case 16 (de novo glioblastoma). CD133-positive staining surrounds areas of necrosis with few Ki 67–positively stained cells. Bar = 100 μm.  

E: Percentage of CD133-positive glioma cells per tumor area in 70 patients with different grades of gliomas. Closed squares and error bar represent the mean ± SD of each group.
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since only CD133-positive cells from brain tumor biopsy material were able to initiate brain cancer in a mouse model.27 CD133 is a 120-kD 5-transmembrane cell-surface protein, previously known as a hematopoietic stem cell marker.18 Several studies have recently demonstrated the immunohistochemical detection of CD133 antigen in glioma cells on frozen or paraffin sections.7,17,22,35 However, observations of CD133 expression are inconsistent among these reports. Hermansen et al.11 suggested that this inconsistency is due to the use of different CD133 antibody clones and different detection systems. Most CD133-related experiments have used 2 monoclonal antibodies, AC133 and AC141, which target poorly characterized glycosylated epitopes of uncertain specificity.8,18

In the present study, we demonstrated clear immunoreactivity for CD133 in paraffin sections from patients with gliomas using a specific antibody and the CSAII method. The primary antibody, a C24B9 clone, used in the present study was generated against a peptide corresponding to a region surrounding Asp562 of human CD133. The C24B9 clone recognizes a nonglycosylated extracellular epitope of CD133 and was suggested to be a suitable antibody for identifying brain tumor stem cells in gliomas.4 The CSAII system is a biotin-free highly sensitive staining procedure based on an indirect immunohistochemical method employing amplification of peroxidase-catalyzed deposition of a fluorescein-labeled phenolic compound. Using this antibody and this system, we identified specific immu-
noreactivity for CD133 antigen on the cell membrane but not in the nuclei of glioma cells. CD133-positive glioma cells usually appeared in clusters and were often located close to tumor blood vessels and were occasionally seen in pseudopalisade formations delineating necrosis. The frequency of CD133 expression in the present study is consistent with those studies using flow cytometry.\(^{3,20}\) Taken together, the present immunohistochemical observations constitute comprehensive data on the detection of CD133 antigen in paraffin sections and thus allow comparison between primary and recurrent tumors after radiotherapy and chemotherapy.

The current glioma stem cell concept suggests that gliomas may originate from neural stem cell populations.\(^{25,34}\) Gliomas develop as a result of genetic alterations that accumulate with tumor progression. Glioblastomas can develop either de novo with no prior evidence of a lower-grade glioma or through malignant progression from a low-grade glioma (that is, secondary glioblastoma).\(^{16}\) However, the possibility remains that the clinical

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**Fig. 2.** Increased frequency of CD133 in recurrent de novo glioblastomas after radiotherapy and chemotherapy.  
**A:** Immunohistochemistry for CD133 in sections from primary tumors after EBRT and GKS in Case 7. Arrowhead designates CD133-positive cells. Bar = 100 μm.  **B:** Immunohistochemistry for CD133 in sections from recurrent tumors after EBRT and GKS in Case 7. Bar = 100 μm.  **C–H:** Case 9. Contrast-enhanced axial MR images at the initial diagnosis (C), at recurrence after resection and irradiation (D), and in the terminal stage (E). There is no CD133 immunopositivity in the primary surgery specimen (F), but extensive immunopositivity is seen in pseudopalisade formations in the section obtained from the remote-site recurrence (red arrow in E) at autopsy (G and H). Bar = 100 μm (F–H).
diagnosis of de novo glioblastomas may include secondary glioblastomas if those tumors rapidly progressed from precursor lesions that escaped clinical diagnosis. These 2 glioblastoma subtypes constitute distinct disease entities that evolve through different genetic pathways and affect patients at different ages. In our present study, CD133 expression on glioma cells was mostly confined to de novo glioblastomas and was not observed in lower grade gliomas, including anaplastic oligodendrogliomas. Secondary glioblastomas rarely expressed CD133 antigen after malignant progression. Beier et al. also showed that CD133-positive cancer stem cells determined by flow cytometry...
are maintained in only a subset of de novo glioblastomas and that CD133-negative glioblastoma cells obtained from secondary glioblastomas are also tumorigenic in nude mice. Because CD133 expression is restricted to de novo glioblastomas, secondary glioblastoma as well as lower-grade gliomas may be derived from different glioma stem cells. It remains to be determined which enrichment marker can be used to detect glioma stem cells in lower grade gliomas and secondary glioblastomas.

Cancer stem cells have been proposed to play a key role in the mechanism underlying resistance to anticancer treatment of malignant gliomas. Bao et al. showed a glioma stem cell subpopulation to promote radioresistance in glioblastoma cells in culture and ex vivo. However, it is currently unknown whether a cancer stem cell that can initiate a tumor after transplantation is the same as a cancer stem cell that can cause recurrence after anticancer treatment. We found that CD133-positive glioma cells showed expansion in tissue sections of local tumor recurrence after radiation and chemotherapy in cases with de novo glioblastomas while being rare in primary sections obtained prior to adjuvant treatment. Autopsy cases with de novo glioblastomas with multiple disseminations showed extensive distributions of CD133-positive glioma cells in sections obtained from both local recurrence and remote-site recurrences. In contrast, secondary glioblastomas rarely expressed CD133 antigen after malignant progression following irradiation. Similarly,

**Fig. 4.** Effects of high-dose radiation on tumor blood vascular density. A and B: Immunohistochemistry for factor VIII in sections obtained during the primary surgery (A) and the second surgery 26.2 months after high-dose radiation (B) in a patient with glioblastoma (Case 5). Bar = 100 µm. C: Graphs showing tumor blood vessel density before and after high-dose radiation (GKS plus EBRT, Cases 1–8) and EBRT alone (Cases 11–20) in de novo glioblastomas with local failure. Tumor blood vessel density was significantly decreased in sections obtained after high-dose radiation, while specimens obtained after EBRT alone did not differ significantly from the primary surgery. Closed squares and error bars represent the mean ± SD of each group. ^p < 0.005; n.s. = not significant versus primary tumor by paired t-test.
no CD133-positive tumor cells were detected in anaplastic oligodendrogliomas after irradiation treatment. Our data suggest that CD133-positive glioma stem cells can survive radiation and chemotherapy and thereby contribute to treatment failure in de novo glioblastomas. Ki 67 indices of CD133-positive glioma cells were significantly increased in recurrent glioblastomas after radiation and chemotherapy as compared with those in primary tumors. These data indicate that CD133-positive glioma stem cells change to a proliferative cancer stem cell phenotype in de novo glioblastomas during the development of recurrence after radiation and chemotherapy. Increased rates of tumor growth during gaps in radiotherapy (that is, accelerated repopulation) have been described in head and neck epithelial tumors. Phillips et al. showed that the number of breast cancer–initiating cells in culture increased after short courses of fractionated irradiation through activation of Jagged-1 and Notch-1 signaling pathways. Vlashi et al. also demonstrated that the number of cells double positive for cancer stem cell markers and Ki 67 tripled 72 hours after sublethal doses of radiation in tumor-bearing mice, suggesting that accelerated tumor repopulation may

Fig. 5. Proliferative activity in CD133-positive glioma cells by double immunostaining for CD133 and Ki 67. A and B: Double immunostaining for CD133 (brown) and Ki 67 (purple) on sections from primary (A) and recurrent (B) tumors in a patient with glioblastoma (Case 8). Green arrows indicate Ki 67–positive and CD133-negative cells and red arrows indicate Ki 67 and CD133 dual-positive cells. Bar = 20 μm. C: Ki 67 indices of CD133-positive glioma cells in primary and recurrent tumors. Closed squares and error bars represent the mean ± SD of each group. *p < 0.00000005 versus primary tumor by unpaired t-test. D: Ki 67 indices of CD133-negative glioma cells. **p < 0.02 versus primary tumor by paired t-test.
be derived from the cancer stem cell compartment. Because histological sections of recurrent tumors in the present study were obtained several months after radiotherapy and chemotherapy, a switch in proliferation of glioma stem cells is not a temporary but rather a constitutive event that may play a key role in the mechanisms producing radioresistance and expansion of CD133-positive glioma stem cells in de novo glioblastomas.

Glioblastomas are highly vascularized brain tumors and are considered to be attractive targets for antiangiogenic therapies. Calabrese et al. demonstrated that CD133-positive/nestin-positive glioblastoma cancer stem cells are located close to tumor capillaries and are maintained within vascular niches that mimic the neural stem cell niche. Hammondzumyan et al. showed that medulloblastoma stem cells residing in the perivascular niche survive radiation via activation of the PI3K/Akt pathway. If the brain tumor microvasculature forms a niche that is critical for the maintenance of cancer stem cells, targeting the tumor microvasculature to disrupt stem cell maintenance could be a promising approach to the treatment of malignant gliomas. In the present study, the number of tumor blood vessels was significantly reduced after high-dose radiation delivered by EBRT and GKS but not after EBRT alone. Although high-dose radiation effectively induces tumor necrosis and tumor vascular damage in de novo glioblastomas, the frequency of CD133-positive glioma cells was apparently greater in sections obtained after high-dose radiation than in those subjected to EBRT alone. These observations indicate that CD133-positive glioma stem cells can survive and cause recurrence even in the absence of intimate contact with tumor vasculature after high-dose radiation. Sakariassen et al. reported that highly infiltrative gliomas with a stem cell phenotype showed angiogenesis-independent growth in an early passage after xenotransplantation. Enhanced tumor cell invasion after antiangiogenic therapy has also been reported in glioblastoma xenografts. The CD133-positive glioma stem cells that expand after radiotherapy and chemotherapy may also switch to a highly invasive phenotype with tumor cells being dispersed throughout the brain in close proximity to resident normal blood vessels. Further characterizing the glioma stem cells that expand after radiation and chemotherapy is anticipated to elucidate the mechanisms of resistance in malignant gliomas, possibly allowing the current limitations in the treatment of these tumors to be overcome.

Conclusions

Expression of CD133 on glioma cells was confined to de novo glioblastomas but was not observed in lower-grade gliomas. The frequency of CD133-positive glioma stem cells was significantly increased in recurrent de novo glioblastomas after radiotherapy and chemotherapy. Our results indicate that CD133-positive glioma stem cells can survive, change to a proliferative cancer stem cell phenotype, and cause recurrence in de novo glioblastomas despite tumor vascular damage after radiotherapy and chemotherapy. This could be an essential factor limiting the effectiveness of concurrent radiochemotherapy for malignant gliomas. Further investigation focusing on glioma stem cells is anticipated to elucidate the mechanisms of glioma recurrence.

Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contributions to the study and manuscript preparation include the following. Conception and design: Tamura, Aoyagi. Acquisition of data: all authors. Analysis and interpretation of data: all authors. Drafting the article: Tamura, Aoyagi. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Tamura. Statistical analysis: Tamura, Aoyagi. Administrative/technical/material support: Ando, Yamamoto, Ohno. Study supervision: Aoyagi, Ohno.

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