

Transforming growth factor- β and stem cell markers are highly expressed around necrotic areas in glioblastoma

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Abstract Invasion into surrounding normal brain and resistance to genotoxic therapies are the main devastating aspects of glioblastoma (GBM). These biological features may be associated with the stem cell phenotype, which can be induced through a dedifferentiation process known as epithelial-mesenchymal transition (EMT). We show here that tumor cells around pseudopalisading necrotic areas in human GBM tissues highly express the most important EMT inducer, transforming growth factor (TGF- β), concurrently with the EMT-related transcriptional factor, TWIST. In addition, the stem cell markers CD133 and alkaline phosphatase (ALPL) were also highly expressed around necrotic foci in GBM tissues. The high expression of TGF- β around necrotic regions was significantly correlated with shorter progression-free survival and overall survival in patients with GBM. High expression of stem cell markers, ALPL, CD133, and CD44 was also correlated with poor outcomes. These results collectively support the hypothesis that tissue hypoxia induces the stem cell phenotype through TGF- β -related EMT and contributes to the poor outcome of GBM patients.

Keywords Glioma · Microenvironment · Hypoxia · TGF- β · Epithelial-mesenchymal transition · EMT

Introduction

Gliomas are classified according to histopathological features that distinguish three cellular lineages, astrocytic, oligodendrocytic, and ependymal, and four malignancy grades, grades I to IV according to the criteria established by the World Health Organization (WHO) [1]. The most malignant form of gliomas, grade IV glioblastoma (GBM), is supposed to originate from oligodendrocyte precursor cells [2]. The most devastating and refractory aspects of GBM are the highly invasive nature that prevents curative tumor resection and the resistance to apoptosis-inducing therapies. Individual tumors with the same morphology likely have different refractory features that are responsible for the markedly heterogeneous survival periods [3]. According to The Cancer Genome Atlas sub-classification of GBM, the mesenchymal subtype frequently expresses neural stem cell markers and is associated with an aggressive phenotype [4–6]. Non-mesenchymal subtypes usually acquire the gene expression pattern of the mesenchymal subtype at recurrence after chemotherapy and radiotherapy [6], indicating that phenotypic transition may depend on environmental signals.

A prominent characteristic of the GBM microenvironment is hypoxia, which is caused by abnormal structure, function, and distribution of neo-angiogenesis and aberrant induction of the intravascular coagulation cascade [7, 8]. A hypoxic microenvironment is generally regarded as a potent inducer of epithelial-mesenchymal transition (EMT) which induces polarized epithelial cells to undergo multiple epigenetic changes that result in dedifferentiation of cells and acquisition of stem cell features [11–15]. The EMT is mainly mediated by transforming growth factor- β (TGF- β) among many other signaling pathways [9, 10]. TGF- β family members also play an important role in the

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maintenance of pluripotent stem cells and cancer stem cells [16]. In addition, a hypoxic microenvironment in tumor tissues leads to recruitment of circulating or residential myeloid cells, such as macrophages or microglia, into the tumor stroma [16, 17]. These cells release high levels of TGF- β , which triggers upregulation of transcription factors necessary for EMT such as TWIST or Snail and further progression of EMT [16–21]. Indeed, the phenotypic transition to the mesenchymal subtype of GBM may be directly induced by TGF- β [22].

We hypothesize that hypoxia induces stem cell properties in GBM cells through TGF- β -mediated EMT, which contributes to treatment resistance and poor outcomes. Such a correlation has not been demonstrated in human GBM tissues. To address this issue, we performed an integrated histological and molecular analysis of EMT-related factors such as TGF- β and TWIST, and we analyzed the expression of stem cell markers in relation to necrotic areas in GBM tissues.

Materials and methods

Patients and tissue specimens

We performed a retrospective study of 80 patients with GBM. All tissue samples were obtained from patients treated at the Chiba University Hospital at the time of the patient's first surgery. Two independent neuropathologists confirmed the histopathological diagnoses of all specimens according to the criteria established by WHO in 2007. All patients with GBM were treated with 60 Gy local-field irradiation and concurrent temozolomide. The chemotherapy protocol was administered according to Stupp's regimen, and the maintenance chemotherapy was continued for a median of 1.5 years. Written informed consent was obtained from each patient. Brain magnetic resonance images of all patients were obtained with and without gadolinium enhancement to assess the infiltrative or disseminated areas during the survival period.

Immunohistochemical analysis

For immunohistochemical analyses of gliomas, paraffin-embedded samples were sectioned at a thickness of 6 μ m and mounted on microscopic slides. The primary antibodies used in this study were: anti-TGF- β 1 antibody (1:200, R&D Systems, Minneapolis, MN), anti-alkaline phosphatase (ALPL) antibody (1:100, Abcam, Inc. Cambridge, UK), anti-CD133 antibody (1:20, Miltenyi Biotec, Germany), anti-CD44 antibody (1:100, Novocastra Lab-

oratories, Ltd., Newcastle, UK), anti-TWIST antibody (1:100, Abcam), anti-IDH1-R132H antibody (1:100, MBL Co., Ltd, Nagoya, Japan), and anti-O6-methylguanine-DNA methyltransferase (MGMT) monoclonal antibody MT3.1 (1:200, Chemicon, Inc., Temecula, CA, USA). Heat-induced epitope retrieval was performed by microwaving the sections in 10 mM EDTA buffer, pH of 7.2. The samples were incubated overnight with the antibodies in the same buffer, followed by incubation with biotinylated secondary antibody (1:500, DAKO, Tokyo, Japan). The bound antibodies were visualized with the avidin–biotin–peroxidase complex method and diaminobenzidine tetrachloride (Santa Cruz Biotechnology, Santa Cruz, CA). Sections were counterstained with hematoxylin. A positive staining result was considered a tumor in which >10 % of cells had cytoplasmic, membranous or nuclear staining.

Histological analysis related to pseudopalisading necrosis

The pseudopalisading necrosis is shown to represent a hypoxic areas [23]. For immunohistochemical analysis, the positive rates were calculated as the percent of cells positive for the marker. We selected two representative areas both in the pseudopalisading necrotic areas and non-necrotic areas, and calculated the positive rate separately. When the positive rate in the necrotic area was 10 % or more, the case was judged as “necrotic positive” irrespective of the positive rate of non-necrotic areas. When the positive rate in the necrotic area was below 10 % and that in the non-necrotic area was 10 % or more, the case was judged as “non-necrotic positive”. Therefore, some necrotic positive cases may have a higher positive rate of the non-necrotic area than that of the necrotic area.

Statistical analysis

The survival periods of patients with GBM were calculated. Progression-free survival (PFS) was calculated from the date of initial surgery until the first sign of radiological progression, death, or last follow-up. Overall survival (OS) was also calculated from the date of initial surgery until the date of death or last follow-up. The Kaplan–Meier method was used to estimate the survival rates, and the Cox–Mantel log-rank test was applied to compare the survival differences among the patients. Analyses were performed using StatView software (SAS Institute Inc., Cary, NC). Multivariate analysis was performed with the commercially available software using the Cox proportional hazards regression model (SPSS, Inc., Chicago, IL).

Results

Clinical course of the GBM patients

Eighty patients with histologically confirmed, newly diagnosed GBM from 2007 to 2014 were included in this study. Characteristics of the patients are summarized in Table 1, and are similar to those of previous reports. Patients were equally distributed between older and younger than 60 years of age and by sex. Karnofsky performance status (KPS) score below 70 was observed in 45 patients (56 %). Total resection was accomplished in 34 patients (43 %). MGMT protein expression that was judged to be 10 % or more was observed in 34 of the 75 cases with tissue available (45 %). IDH1 status was also determined with immunohistochemical analysis using anti-IDH1R132H antibody, which showed that five cases (6 %) were positive for the IDH1-mutant protein. The median PFS was 16 months, and the median OS was 22 months. Marked heterogeneity in OS was observed among the patients, with an OS of over 5 years in nine patients and less than 1 year in 18 patients.

TGF- β

Necrotic areas, which are considered to be hypoxic, were observed in all cases. Only pseudopalisading necrosis was considered for evaluation of the cellular response to hypoxia in this study. All the results of immunohistochemical analyses are summarized in Table 2. The result showed positive TGF- β expression in 71 % of the patients (53 among 75 patients examined). The necrotic-positive frequency was 27 % (20 of 75 cases) and the frequency of non-necrotic positive was 44 % (33 of 75 cases) (Fig. 1a).

Table 1 Patient characteristics

Characteristics	N (%)
Sex	
Male	40 (50)
Female	40 (50)
Age	
<60	39 (49)
60 \leq	41 (51)
KPS	
70 \leq	37 (46)
<70	43 (54)
Surgery	
Total resection	34 (43)
Non-total resection	46 (57)
IDH1 gene status	
Wild-type	70 (93)
Mutated	5 (7)

The mean positive rate for TGF- β in the necrotic-positive cases was 34.3 ± 19.2 %, whereas that of the non-necrotic regions was 18.6 ± 8.3 % ($p = 0.0027$, unpaired t test) (Fig. 1b). This result indicates that TGF- β expression is higher in hypoxic areas than in non-hypoxic areas in some GBM patients.

TWIST

TWIST is a strong inducer of EMT, which is directly correlated with stem cell properties. Immunohistochemical analysis showed that 47 % of the patients had tumors that were positive for TWIST (35 of 75 patients examined). Similar to TGF- β , the positive rate of TWIST tended to be higher in necrotic areas than in non-necrotic areas (24.1 ± 5.5 % vs. 10.6 ± 5.5 %) without statistical significance ($p = 0.1234$, unpaired t -test). This is likely in part because the number of positive cases was small compared with the number of cases that expressed TGF- β . Expression of TWIST was a significant factor for short PFS ($p = 0.0273$) but not for OS.

Relationship between stem cell markers and necrosis

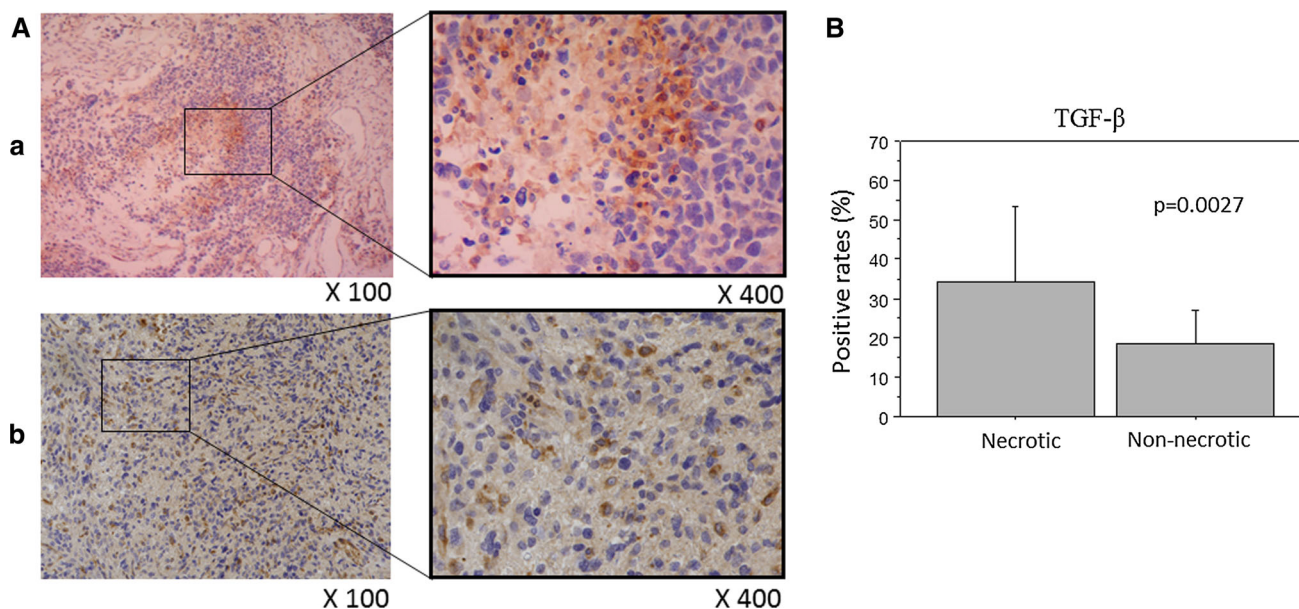
Because necrotic areas in GBM tissues were enriched with TGF- β and TWIST, we also analyzed the expression of stem cell markers. The stem cell markers CD133, CD44, and the pluripotent stem cell marker ALPL were examined. ALPL is highly expressed in the embryonic inner cellular mass and is traditionally used as a pluripotent stem cell marker [15–18]. Immunohistochemical analysis showed that CD133 was positive in 52 % of the patients (33 of 64 patients examined) (Fig. 2a). The positive rate of CD133 was significantly higher in necrotic areas than in non-necrotic areas (36.9 ± 16.3 % vs. 22.0 ± 11.4 %) ($p = 0.0047$, unpaired t -test) (Fig. 2b). ALPL was positive in 55 % of the patients (35 of 64 patients examined). The positive rate was also significantly higher in necrotic areas than in non-necrotic areas (34.1 ± 15.1 % vs. 21.3 ± 9.5 %) ($p = 0.0106$, unpaired t -test) (Fig. 2b). In contrast, CD44 was expressed in all GBM tissues examined, and the immunostaining pattern was categorized as moderate ($n = 36$, 51 %) or intense ($n = 34$, 49 %). However, the staining pattern was not correlated with necrotic areas.

Patient survival according to TGF- β expression in necrotic areas

Patients with high TGF- β expression tended to have shorter survival than those without TGF- β expression, but the difference was not statistically significant ($p = 0.1211$). TWIST expression was also not significantly associated

Table 2 Summary of the cell-positivity rates in immunohistochemical analyses

	Non-necrotic areas (%)	Necrotic areas (%)	p-value
TGF- β	18.6 \pm 8.3	34.3 \pm 19.2	0.0027
TWIST	10.6 \pm 5.5	24.1 \pm 5.5	0.1234
Alkaline phosphatase	21.3 \pm 9.5	34.1 \pm 15.1	0.0106
CD133	22.0 \pm 11.4	36.9 \pm 16.3	0.0047
CD44	100 %: Moderate staining 51 %, intense staining 49 %		

**Fig. 1** Transforming growth factor- β (TGF- β) immunohistochemistry. **a** Representative results of a necrotic positive case in which positive cells are clustered around a necrotic area (**a**), and a non-necrotic positive case in which positive cells are mainly located in an

area distant from necrosis (**b**). **b** Bar graph comparing the positive rates of TGF- β expression between necrotic and non-necrotic areas ($p = 0.040$)

with patient survival (data not shown). We next compared the patient survival among three classes of TGF- β expression related to necrotic areas: negative (both necrotic and non-necrotic areas showed staining of 10 % or less), non-necrotic positive (only non-necrotic areas were positive), or necrotic positive (necrotic areas were positive). Twenty-two cases were negative, 33 cases were non-necrotic positive, and 20 cases were necrotic positive. The patients who were necrotic positive showed significantly shorter survival than those who were non-necrotic positive or with negative TGF- β expression ($p = 0.0028$ for PFS and $p = 0.0134$ for OS, necrotic positive vs others, log-rank test) (Fig. 3). Expressions of ALPL, CD133, and CD44 each was a poor prognostic factor for survival irrespective of expression patterns related to necrosis. ALPL was significant both for OS and PFS ($p = 0.0102$ and $p = 0.0447$, respectively) and CD133 was significant only for OS ($p = 0.0207$). The intense CD44 immunostaining was a significant factor both for a shorter PFS and a shorter OS ($p = 0.0089$ and $p = 0.0119$, respectively).

Multivariate analysis of survival

To confirm the prognostic value of expression levels of stem cell markers, we performed multivariate analysis of survival of 64 patients who had full immunohistochemical data among 80 GBM patients included in this study (Table 3). Known prognostic factors such as age (≥ 60 years), KPS (< 70), extent of surgical resection (non-total resection), and MGMT protein expression were also included in the analysis. The stem cell markers ALPL and CD133 were also included. An age of ≥ 60 years and KPS of < 70 did not significantly affect the survival period. Non-total resection was an independent factor for poor prognosis both in PFS and OS (HR 3.376, $p = 0.0290$ and HR 3.276, $p = 0.0275$, respectively). Among the molecular factors, only ALPL was a significant factor for OS but not for PFS (HR 2.967, $p = 0.0384$ and HR 2.734, $p = 0.0725$, respectively). No other molecular factors were significant in the multivariate analyses including high TGF- β expression around necrosis.

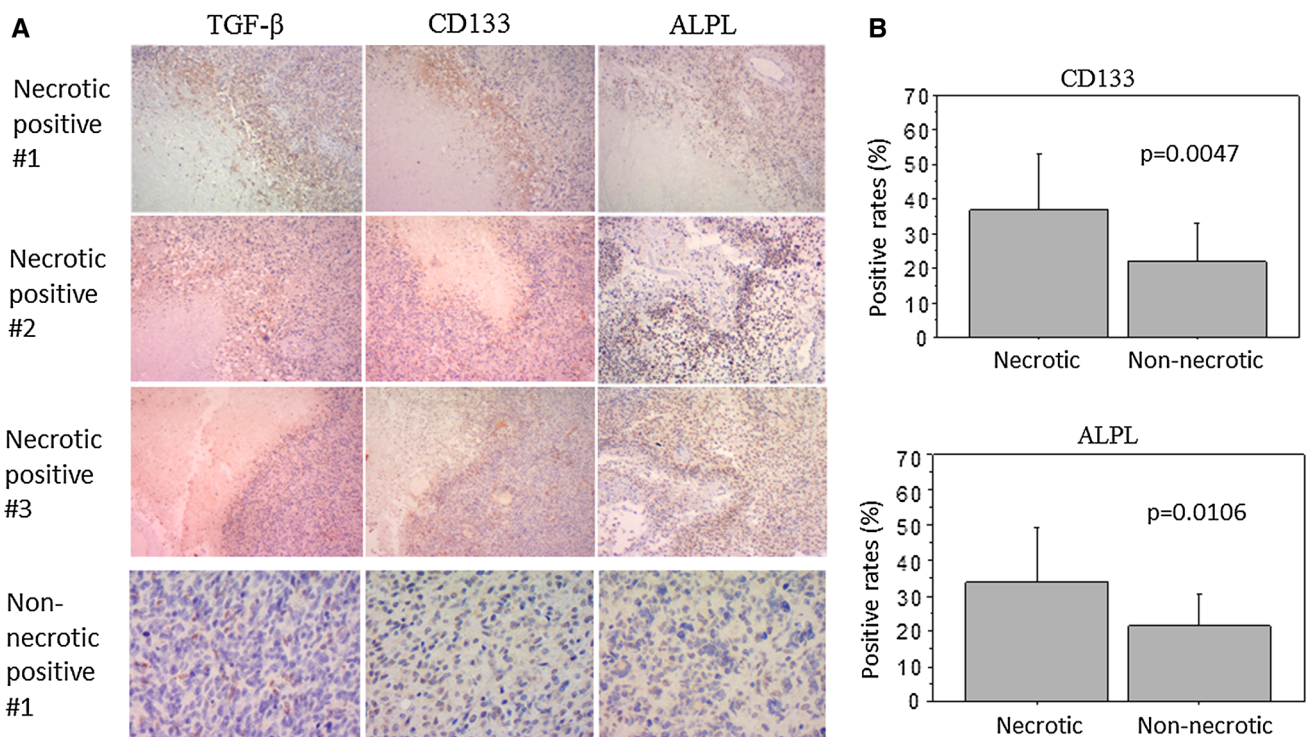


Fig. 2 Concurrent expression of transforming growth factor-β (TGF-β), CD133, and alkaline phosphatase (ALPL) around necrotic areas (a). CD133 and ALPL expression was higher in necrotic areas than in non-necrotic areas ($p = 0.007$ and 0.045 , respectively) (b)

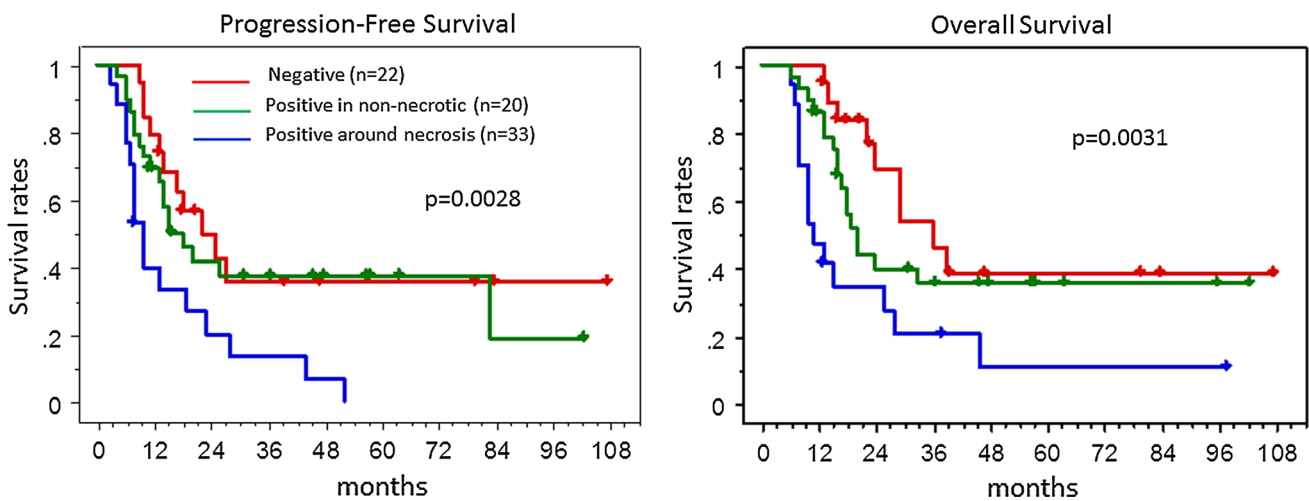


Fig. 3 Kaplan-Meier analyses according to the mode of transforming growth factor-β (TGF-β) expression. Patients with tumors expressing high levels of TGF-β around necrotic areas lived significantly shorter than those with the tumors expressing low levels of TGF-β around necrotic areas

Discussion

We show in this report that high expression of TGF-β, TWIST, and stem cell markers is more frequently observed around necrotic areas than non-necrotic areas in human GBM tissues. Patients with positive immunostaining of TGF-β tended to have shorter survival than those without positive TGF-β staining without a statistical significance

[24]. This tendency has been confirmed in the patients with necrotic positive of TGF-β who survived significantly shorter than those without the TGF-β expression pattern. TGF-β is frequently positive in GBM (71 % in this study) as a result of various signal transduction pathways [24]. Only high expression of TGF-β around hypoxic areas may be correlated with poor survival, which is mainly caused by the acquisition of stem cell properties through EMT. The

Table 3 Multivariate analysis including stem cell markers

Factors	Progression-free survival (PFS)		Overall survival (OS)	
	Hazard ratio (95 % CI)	<i>p</i> -value	Hazard ratio (95 % CI)	<i>p</i> -value
Age (≥ 60 years)	1.122 (0.494–2.561)	<i>p</i> = 0.8231	1.281 (0.533–3.041)	<i>p</i> = 0.5892
KPS (< 70)	1.485 (0.627–4.289)	<i>p</i> = 0.3587	1.834 (0.628–5.244)	<i>p</i> = 0.2373
Surgery (non-total)	3.376 (1.283–9.527)	<i>p</i> = 0.0290	3.276 (1.121–9.412)	<i>p</i> = 0.0275
MGMT	1.884 (0.829–5.673)	<i>p</i> = 0.1663	1.724 (0.638–5.226)	<i>p</i> = 0.2476
TGF- β	2.278 (0.875–4.855)	<i>p</i> = 0.0774	2.013 (0.537–3.268)	<i>p</i> = 0.1165
TWIST	1.853 (0.798–3.546)	<i>p</i> = 0.1278	2.462 (0.875–4.283)	<i>p</i> = 0.0863
CD133	1.573 (0.599–3.352)	<i>p</i> = 0.5612	2.151 (0.793–4.326)	<i>p</i> = 0.1218
ALPL	2.734 (1.235–4.736)	<i>p</i> = 0.0725	2.967 (1.248–5.187)	<i>p</i> = 0.0384

negative result in multivariate analysis would be due to a low statistical power of the small patient cohort. These results collectively suggest that a hypoxic microenvironment is correlated with EMT induction through TGF- β expression and that the resulting stem cell phenotype in GBM tissues may be a main cause of treatment resistance and poor outcomes.

Hypoxia is observed not only during the natural course of glioma progression but also in response to treatment of the glioma including radiation therapy and a targeted therapy such as bevacizumab. These therapeutic modalities are toxic to the vascular endothelial cells and shrink the vascular system, leading to further hypoxia in the tumor tissues. Glioma cells that have survived irradiation possess gene expression patterns that are enriched in EMT-inducing molecules, which endows a radioresistance to the tumor cells [25, 26]. DNA damage responses in GBM cells promote migration and invasion of the tumor cells through EMT with enhanced expression of TGF- β , hypoxia-inducible factor-1 α , vascular endothelial growth factor (VEGF), epidermal growth factor, or hepatocyte growth factor [27–32]. Recently, Mahabir et al. showed that the radiation-related induction of EMT in malignant gliomas is accelerated by TGF- β derived from mesenchymal cells in the tumor microenvironment [32]. Although hypoxia is a main inducer of the TGF- β -related EMT, a direct correlation of hypoxia and TGF- β has been scarcely evidenced in human GBM. The present data support the hypothesis that TGF- β induced by hypoxia plays a crucial role in the possible acquisition of invasive phenotype following radiation therapy.

Treatment with bevacizumab, which targets VEGF, initially restores abnormal distribution and high permeability of vasculature via a process called the normalization phase [33]. With prolonged treatment with bevacizumab beyond the vascular normalization phase, the tumor develops progressive hypoxia [33]. Hypoxia is an important environmental stimulus that directly or indirectly promotes the stem cell phenotype [34–37]. First, hypoxia induces DNA hypomethylation, which is the epigenetic

background of stem cells [38, 39]. Second, hypoxia induces glioma cells to release hypoxia inducible factor-1 α and VEGF, which attract myeloid cells from bone marrow into glioma tissues [16, 17]. The recruited myeloid cells release multiple growth factors such as interleukin-6, interleukin-10, and matrix metalloproteinases, as well as TGF- β [16, 17, 40]. TGF- β also recruits mesenchymal stem cells into the glioma tissues, which contributes to further malignant progression of GBM [9, 21, 41]. This vicious hypoxia-related cycle may be a reason that bevacizumab is not effective in prolonging OS in newly diagnosed GBM patients.

The clinical picture of glioma is quite heterogeneous, even in the same histological category and grade. Such a phenotypic diversity in glioma tissues is partly due to various microenvironmental factors as well as intrinsic genetic alterations. We found that the essential EMT-inducing factors TGF- β and TWIST are highly expressed around necrotic areas in relation to high expressions of stem cell markers. Anti-VEGF therapy such as bevacizumab may be candidates for modulating the glioma hypoxic microenvironment to enhance the efficacy of radiotherapy and to obtain a better outcome for GBM patients.

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