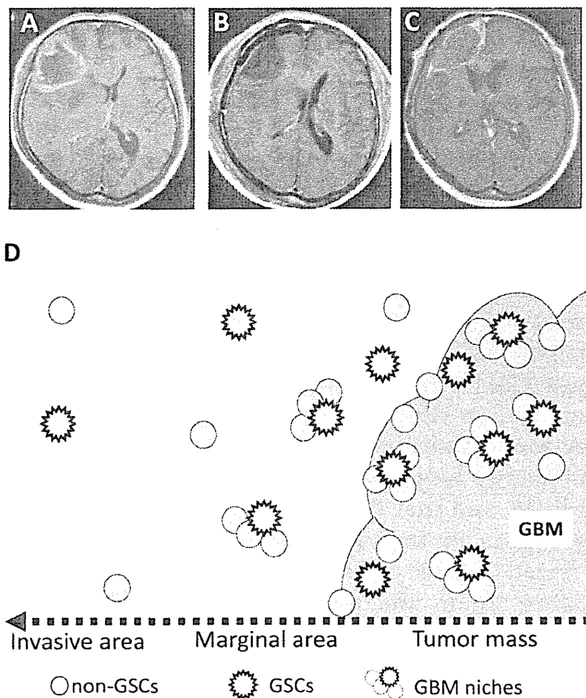

Fig. 1 Recurrence of GBM. A–C: Contrast-enhanced MRI of a patient with GBM in the right frontal lobe obtained before surgery (A), after treatment with TMZ and radiotherapy (B), and at recurrence (C). D: Schematic drawing of GBM cell invasion into the deep brain. Although the GBM tumor mass is removed, invaded cells remain in the brain. Clusters of GSCs and non-GSCs form specific environments (GBM niches) with some extracellular matrix and secreting molecules. GBM recurs in the periphery of the removal cavity (marginal area) where some GSCs in GBM niches are resistant to chemo-radiotherapy and survive. GSCs without GBM niches in the invasive area die, stop growing, or grow below the detection level on MRI. GBM: glioblastoma multiforme, GSCs: glioblastoma stem cells, MRI: magnetic resonance imaging, TMZ: temozolomide.

in cases with complete postoperative disappearance of the enhanced lesion (Fig. 1A–C).[27] This suggests that migrated tumor cells far from the tumor mass are killed by conventional chemo-radiotherapy or that the growth of residual tumor cells is below the level of detection. Thus GSCs around the removal cavity may be able to escape the effects of current multimodal therapies and the recurrence of GBM may be attributable to the persistence of surviving dormant GSCs in GBM niches around the removal cavity (Fig. 1C, D).

Despite extensive efforts to cure GBM patients, curative therapies remain elusive. Beier et al.[8] who summarized accumulated information on the chemoresistance of GBMs concluded that the interactions of GSCs and chemotherapy are highly complex and that intrinsic and extrinsic factors are involved. Here we focus on GSCs and GBM niches as therapeutic targets and discuss the need for additive treatments.

GSC markers

According to Singh et al.,[69,70] brain tumor initiating cells are concentrated in the CD133$^+$ but not in the CD133$^-$ fraction. Clinically, CD133$^-$ GBMs are characterized by a lower proliferation index.[6,7] However, the CD133 status alone is not sufficient as a GSC marker. Beier et al.[7] reported that cells from primary GBM contained CD133$^+$ subpopulations that formed spheres, and that cells from GBMs that harbored no CD133$^+$ cells grew adherently, and that CD133$^-$ tumor cells could initiate tumors and fulfilled stem-cell criteria. Chen et al.[15] had shown that some CD133$^-$ cells were more primitive than CD133$^+$ cells and that CD133$^-$ cells could produce CD133$^+$ and CD133$^-$ cells. Nishide et al.[56] established induced GSCs (iGSCs) derived from mouse neural stem cells (NSCs). They deleted CD133-expressing cells by tamoxifen-dependent Cre activation and obtained cells that could form GBM. They concluded that CD133 expression was not required for the tumorigenesis of GSCs in nude mice.

While CD133 is one of the markers of GSCs, it is not sufficient for their purification. Other markers used for the detection of GSCs are CD15/SSEA-1/LewisX, A2B5, L1CAM, integrin alpha 6, and CXCR4.[1,47,57,72,83] Kijima et al.[41] who reported that CD166/activated leukocyte cell adhesion molecule (ALCAM) was highly expressed in CD133$^+$ GSCs showed that ALCAM and its soluble isoform are involved in the regulation of glioblastoma invasion and progression.

Another technique used to identify GSCs is utilization of their drug efflux ability through ATP-binding cassette (ABC) drug transporters. Hematopoietic stem cells (HSCs) express high levels of ABCG2, but the gene is turned off in committed progenitors and mature blood cells.[68] These transporters protect HSCs from cytotoxic agents. Cells expressing ABCG2 excrete Hoechst 33342 fluorescent dye; they are detected by fluorescence-activated cell sorting (FACS) as fluorescent dye-negative cells. Stem cells are concentrated in this small unstained population and this cell fraction is referred to as the side population (SP). The fluorescence-excreting function is inherent in normal somatic stem cells and CSCs. GSCs are concentrated in the SP fraction[34,44] and SP cells are different from non-SP cells in their ability

for self-renewal, tumorigenesity, and resistance to therapy. The drug efflux ability is controlled by several genes of the ABC transporter family and protects CSCs from the effects of chemotherapeutic agents.[20] The ABCG2 gene plays a major role in the control of this function. In the transgenic mouse model a nuclear form of GFP expression under the control of the ABCG2 promoter was detected in the ventricular zone of the developing forebrain and spinal cord where NSCs exist.[58] Patrawala et al.[60] reported that a subpopulation of ABCG2$^-$ cells produced ABCG2$^+$ cells and that both ABCG2$^+$ and ABCG2$^-$ cells are tumorigenic. They concluded that ABCG2 expression primarily identifies fast cycling tumor progenitors and that the ABCG2$^-$ population contains primitive stem-like cancer cells. On the other hand, Broadley et al.[10] documented that doxsorubicin-exposed cells showed a transient increase in SP cells without being enriched for the stem cell phenotype.

Taken together, these findings suggest that GSCs can be enriched by using some cell surface markers and/or the drug efflux ability. However, these techniques are suboptimal if the goal is the purification of *bona fide* GSCs.

Cell origin of GBM

Core signaling pathways, e.g. receptor tyrosine kinase (RTK), p53, and Rb are crucial in clinical studies of glioblastoma.[13] They are also significant for gliomagenesis in both genetically manipulated mouse models and several types of iGSCs transformed from neural lineage cells via the over- and down-regulation of these core pathway genes.[14,26,50,52] To investigate the cell origin of GBMs, we established iGSCs derived from p53-/- NSCs, astrocytes, and oligodendrocyte precursor cells (OPCs). These were transformed by the over-expression of the active form of *H-ras*. While 10 injected iGSCs from NSCs and OPCs formed GBMs in the brains of nude mice, the injection of 10^4 iGSCs from astrocytes was required to form anaplastic astrocytomas, indicating that NSCs and OPCs have a higher potential for gliomagenesis than astrocytes.[32,33] Liu et al.[50] who used a mouse model of p53/Nf1 mutation showed that GBM originates from OPCs and Friedmann-Morvinski et al.[26] who performed p53/Nfl knockdown in mouse brains demonstrated that even mature neurons and astrocytes can induce malignant gliomas. They proposed that upon defined genetic alterations, most differentiated cells in the central nervous system (CNS) undergo dedifferentiation to generate an NSC- or progenitor state, to maintain tumor progression, and to give rise to the heterogeneous populations observed in malignant gliomas. Thus, not only NSCs and OPCs but also mature neurons and astrocytes can be the target of gliomagenesis.[14,26,32,33,50,52]

Characteristics of GSCs

Clinically, GSCs are resistant to conventional chemo- and radiotherapy.[5,20] Residual tumor cells, especially GSCs in GBM niches, lead to recurrence even after primary intensive treatment consisting of surgery and chemo- and radiotherapy.

Bao et al.[2] who studied the radioresistance of GSCs showed that CD133$^+$ glioma cells recovered more quickly from deoxyribonucleic acid (DNA) damage than CD133$^-$ cells by expressing checkpoint kinase (Chk) 1 and 2. Ropolo et al.[67] examined DNA repair in five stem and non-stem glioma cell lines. They found that the population-doubling time was significantly longer for stem- than non-stem glioma cell lines, and that the activation of Chk1 and Chk2 was enhanced in untreated CD133$^+$ compared to untreated CD133$^-$ cells. After irradiation, DNA base excision repair, single-strand break repair, and the resolution of phospho-H2AX nuclear foci, an indicator of double-strand break repair, were not significantly greater in CD133$^+$ than CD133$^-$ cells. They suggested that an elongated cell cycle and enhanced basal activation of checkpoint proteins contribute to the radio-resistance of GSCs and that enhanced DNA repair is not a common feature of these cells.

In GBM, CD133$^+$ cells highly express drug resistance genes and this result in chemoresistance.[9,51] Despite treatment with temozolomide (TMZ), an important anti-GBM drug, some GBM cells survive, leading to tumor recurrence within a year. TMZ kills GBM cells but the ratio of SP cells among residual tumor cells increases.[17] Consequently, although treatment with TMZ plus radiation has extended the mean survival time of GBM patients, this therapy fails to eradicate all GSCs.[75]

Somatic stem cells and CSCs have been identified among slow-dividing and/or dormant cell populations but have not been shown among GSCs.[73] Deleyrolle et al.[21] reported that glioma cells were stained with carboxyfluorescein diacetate succinimidylester (CSFE) and that this fluorescent dye was diluted by cell division. Characteristically, CFSE^{high} cells, i.e., slow-dividing cells, showed a higher expression of stem cell markers and stronger tumor forming more ability than CSFE^{low} cells. This was the first evidence that label-retaining tumor-initiating cell populations within the human GBM-derived glioma sphere are highly tumorigenic GSCs and their findings may help to explain the resistance of GSCs to conventional therapies.

GSCs and hypoxia

According to Pistollato et al.,[64] oxygen tension

controls the expansion of precursors in the human CNS. Low physiological oxygen tension maintains stemness, while higher oxygen tension promotes the differentiation of normal human neural precursors into astrocytes and oligodendrocytes. Hypoxia has critical effects on CSCs.[30,36,38,39,45] With respect to gliomas, it promotes the expression of GSC markers and expands the GSC pool.[4,31,53,54,71,81] Natsume et al.[55] reported that girdin maintains the stemness of GSCs; under hypoxic conditions its expression was up-regulated in parallel with the expression of CD133. Earlier, Pistollato et al.[63] had documented that the intratumoral hypoxic gradient drives stem cell distribution and the expression of MGMT in glioblastoma.

An essential gene regulating the hypoxic condition is hypoxia-inducible factor (HIF). It regulates GBM recurrence and its poor response to treatment and is involved in the poor prognosis of GBM.[37,38,81] Calabrese et al.[11] reported that the stem cell pool in the brain tumor mass physically interacts with the tumor vasculature and endothelial cells. In particular, HIF-1 alpha induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion.[23] HIF regulates the tumorigenic capacity of GSCs and HIF-2 alpha is specifically expressed in GSCs.[49] In addition, HIF-2 alpha expression correlates with the poor survival of glioma patients.[49] Kolenda et al.[43] showed that in addition to the expression of HIF-1 alpha and HIF-2 alpha, the expression of stem cell and chemoresistant markers was increased under hypoxic conditions while Ki-67 was reduced. Together, these findings indicate that hypoxia promotes not only chemoresistance but also stem cell marker expression and slowing of the cell cycle.

GSCs and GBM niches

With respect to HSCs, both perivascular and osteoblastic niches play an essential role in the existence of progenitor and stem cells.[40,82] Doetsch et al.[22] studied neurogenesis in the adult mouse brain. They showed that characteristic microenvironments help NSCs to maintain their ability for self-renewal, multi-lineage differentiation, and infinite proliferation. They designated stem- and proliferative progenitor cells as type B and C cells, respectively, and migrating neuroblasts as type A cells assembled in the subventricular zone where NSCs were in touch with vessels. NSCs reside in the perivascular niche and their self-renewal ability is regulated by this specific microenvironment.[59] Cell-cell- and cell-ECM interactions and interactions among several secreting molecules are important in NSC and GBM niches.[22,25,61]

Hypoxic- and perivascular niches are strongly involved in the initiation, progression, chemotherapy resistance, and recurrence of GBM (Fig. 2A, B).[29] Hypoxia promotes angiogenesis and the migration and expression of stemness genes, resulting in the exacerbation of clinical symptoms due to tumor cell invasion, expansion of the tumor mass and perifocal edema, and it induces resistance to therapy. HIFs are key regulators of vascular endothelial growth factor (VEGF) expression and other hypoxia-responsive genes such as Oct4, Sox2, and Glut1.[29,38,39,49,81] The number of capillaries in GBM tumors correlates with the patient prognosis[48] and CD133^{+} GSCs promote tumor angiogenesis through VEGF.[3]

Zhu et al.[84] reported that endothelial cells create a stem cell niche in GBMs by providing NOTCH ligands that nurture the self-renewal of GSCs. In addition, GSCs recruit endothelial cells and GSCs transdifferentiate into endothelial cells.[3,11,66,79] According to Cheng et al.,[16] GSCs generate vascular pericytes to support vessel function and tumor growth. Like endothelial cells, pericytes are important constituents of GBM niches. Specific microenvironments in hypoxic- and perivascular areas result in the formation of GBM niches. Thus, several genes and molecules in the GBM niches control the maintenance and expansion of GSCs (Fig. 2A, B).

GSCs and GBM niches as treatment targets

The usual targets of chemo- and radiotherapy are rapidly dividing cancer cells because expansion and invasion of the tumor mass into surrounding tissue results in organ dysfunction and local pain. GBM is comprised of heterogeneous cell populations that contain not only rapidly-, slowly- and non-dividing cells but also dormant cells. The fraction of dormant and slow-dividing cells appears to be able to resist chemo-radiotherapy due to drug reflux and DNA repair. Accumulated knowledge regarding GSCs and GBM niches has led to the realization that a paradigm shift is necessary with respect to the targets of GBM treatments. In efforts to eradicate GSCs, the blocking of several key pathways related to the maintenance of stemness has been found to effectively reduce their tumorigenic potential. In fact, inhibition of some pathways, e.g., Sonic hedgehog (Shh), Notch, and Wingless-type (Wnt) attenuated the characteristics of stemness and inhibited the formation of GBMs.[18,24,42]

Differentiation therapy is an additional strategy that targets GSCs. Piccirillo et al.[62] reported that bone morphogenetic protein inhibits the tumorigenic potential of human GSCs. All-trans-retinoic acid (ATRA), a standard drug for the treatment of acute promyelocytic leukemia, was effective against GSCs;

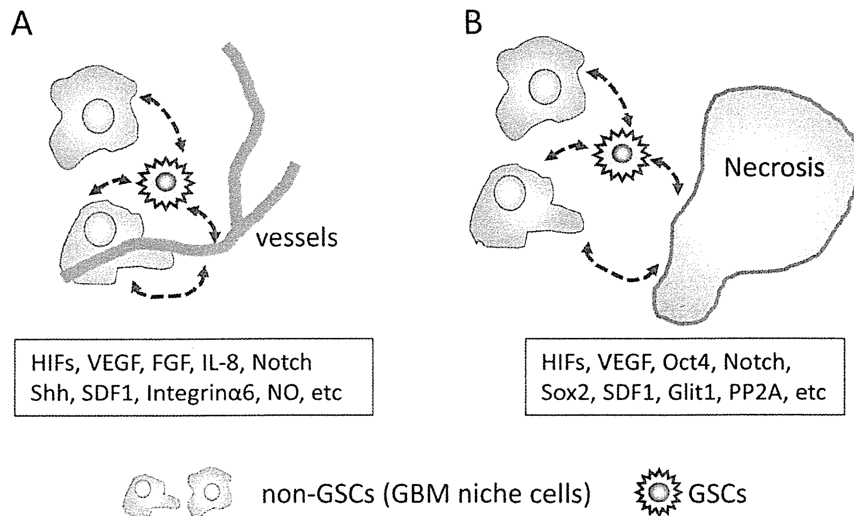

Fig. 2 GBM niches. The stemness of GSCs is maintained by reciprocal signaling in the GBM niche. Perivascular and hypoxic (peri-necrotic) niches are important for GSCs. HIFs and VEGFs are key molecules for the establishment and maintenance of GBM niches. They harbor GSCs, non-GSCs, extracellular matrix, and secreting molecules. Representative genes and molecules are indicated in the box. A: Perivascular niche. Endothelial cells and pericytes in the perivascular area produce molecules for angiogenesis, and also interact with GSCs, non-GSCs, extracellular matrix, and secreting molecules that regulate the expression of genes involved in the maintenance of stemness. B: Hypoxic (peri-necrotic) niche. Hypoxia induces necrosis and regulates the expression of stemness genes. The population of GSCs increases and their proliferation slows. Some GSCs start to invade or enter into dormant state. FGF: fibroblast growth factor, GBM: glioblastoma multiforme, GSCs: glioblastoma stem cells, HIF: hypoxia-inducible factor, VEGF: vascular endothelial growth factor.

it induced differentiation and therapy-sensitizing effects, impaired the secretion of angiogenic cytokines, and disrupted GSCs motility.[12] Hofstetter et al.[35] documented the relationship between hypoxia and the dormancy of GSCs. They showed that protein phosphatase 2A (PP2A) mediates the dormancy of GSCs under hypoxic conditions and that inhibition of PP2A activity results in increased cell proliferation, ATP exhaustion, and the acceleration of P53-independent cell death of hypoxic GSCs.

The perivascular niche is a potential target for GBM treatment. Blocking the SDF-1/CXCR4 pathway prevents or delays tumor recurrence after irradiation by inhibiting the recruitment of monocytes and macrophages that participate in tumor revascularization.[76] In addition, the deletion of vascular pericytes generated from GSCs inhibits tumor growth[16] and a reduction in pro-angiogenic gene expression interrupts perivascular niche formation and results in a decrease in the number of GSCs.[28] Thus, not only specific cells, i.e., endothelial cells and vascular pericytes, but also important genes, i.e., stemness genes and pro-angiogenic genes, are candidate targets in efforts to eradicate GSCs.

Although current conventional GBM treatment strategies can decrease and/or minimize the number of GSCs and GBM niches, they are not curative. Post-treatment, some enhanced lesions indicative of residual tumor disappear on MR imaging scans. Theoretically, both therapy-resistant GSCs and GBM niche cells are minimized at that time, suggesting that nearly "naked" GSCs exist in incomplete GBM niches (Fig. 3). This presents an excellent opportunity for attacking GSCs directly. Besides conventional chemotherapeutic drugs, novel treatment strategies targeting GSCs, and GBM niches may help to cure patients with GBM. The further disruption of GBM niches evacuates GSCs, abolishes their stemness, and induces chemo-radio sensitivity and terminal differentiation. Additionally, due to the specific metabolism and immunoreactivity of GCS, the targeting of GSC-specific cell surface markers may render these cells dormant and/or prove eradicative (Fig. 3).

The development of multi-focal treatment strategies aimed at target cells and target functions and the optimal timing of treatments may improve the survival time and quality of life of GBM patients.

Concluding Remarks

Recently, leukemia has become a curable disease by

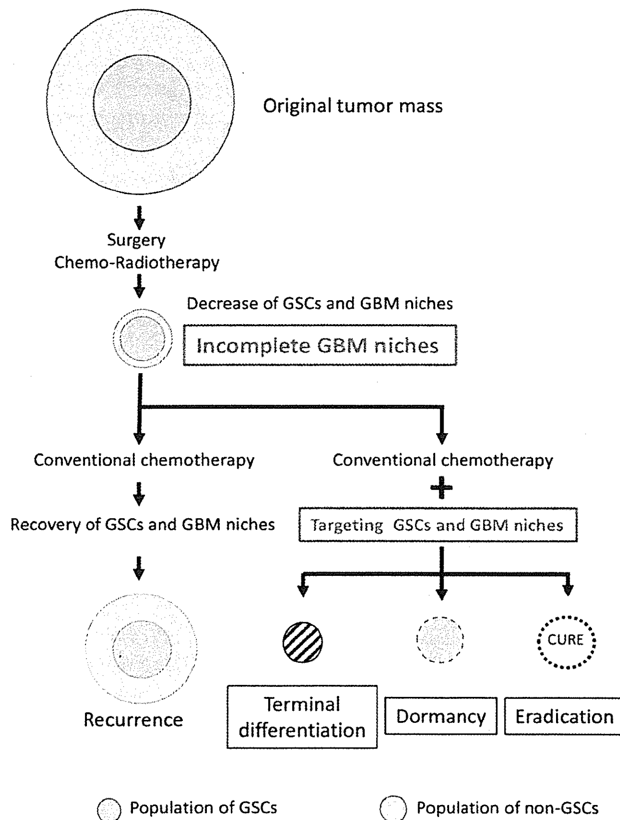

Fig. 3 Additional treatment to eradicate GSCs. Postoperative chemo- and radiotherapy decreases the number of viable GSCs and GBM niche cells, although nearly "naked" GSCs remain in these incomplete GBM niches where suitable microenvironment for supporting GSCs is damaged. Direct attack on the GSCs and/or disruption of GBM niches may result in the terminal differentiation, dormancy, and eradication of GSCs. GBM: glioblastoma multiforme, GSCs: glioblastoma stem cells.

the combination of chemotherapy, radiotherapy, and bone marrow transplantation, but GBM have not. The maximum removal of GBM tissue without eliciting neurological deficits is important for prolonging the survival of GBM patients and for retaining their good quality of life. Actually, the total resection of GBM tumor cells is extremely difficult because they invade into the deep brain.[80] Occasionally, treatment may elicit pancytopenia, radiation necrosis, and the deterioration of cognitive functions in elderly patients. These issues make the radical treatment difficult.

The advent of CSC theory led to fine experiments on GSCs and GBM niches and then showed new insights. An advanced understanding of GSCs and GBM niches can be expected to lead to the development of new therapeutic strategies to cure GBM patients.

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Conflicts of Interest Disclosure

The authors have no personal, financial, or institutional interests in any of the drugs, materials, or devices cited in this article. All authors who are members of The Japan Neurosurgical Society (JNS) have registered online their self-reported COI disclosure statements (available from the JNS website).

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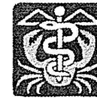

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Prognostic value of isocitrate dehydrogenase 1, O^6-methylguanine-DNA methyltransferase promoter methylation, and 1p19q co-deletion in Japanese malignant glioma patients

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Abstract

Background: To determine the prognostic value of isocitrate dehydrogenase 1 (*IDH1*) mutation, O^6-methylguanine-DNA methyltransferase (*MGMT*) promoter methylation, and 1p/19q co-deletion in Japanese patients with malignant gliomas.

Methods: We studied 267 malignant gliomas, which included 171 glioblastomas (GBMs), 40 anaplastic astrocytomas (AAs), 30 anaplastic oligodendrogliomas (AOs), and 26 anaplastic oligoastrocytomas (AOAs). These malignant gliomas were divided into 2 groups (Group 1: GBM + AA, Group 2: AO + AOA) according to the presence of the oligodendroglioma component. We examined *IDH1* mutation and *MGMT* promoter methylation in each group by direct sequencing and methylation-specific PCR, respectively. We further examined 1p/19q co-deletion in Group 2 by fluorescence in situ hybridization. Survival between groups was compared by Kaplan–Meier analysis.

Results: In Group 1, patients with *IDH1* mutations exhibited a significantly longer survival time than patients with wild-type *IDH1*. However, no significant difference was observed in Group 2, although patients with *IDH1* mutations tended to show prolonged survival. For both Group 1 and Group 2, patients with *MGMT* methylation survived longer than those without this methylation. Further, patients with 1p/19q co-deletion showed significantly better outcome in Group 2.

Conclusions: Our study confirms the utility of *IDH1* mutations and *MGMT* methylation in predicting the prognosis of Group 1 patients (GBM + AA) and demonstrated that *IDH1* mutations may serve as a more reliable prognostic factor for such patients. We also showed that *MGMT* methylation and 1p/19q co-deletion rather than *IDH1* mutations were prognostic factors for Group 2 patients (AOA + AO). Our study suggests that patients survive longer if they have *IDH1* mutations and undergo total resection. Further, irrespective of *MGMT* promoter methylation status, the prognosis of glioma patients can be improved if total resection is performed. Moreover, our study includes the largest number of Japanese patients with malignant gliomas that has been analyzed for these three markers. We believe that our findings will increase the awareness of oncologists in Japan of the value of these markers for predicting prognosis and designing appropriate therapeutic strategies for treating this highly fatal disease.

Keywords: IDH1, MGMT methylation, 1p19q co-deletion, Malignant glioma, Prognosis

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Background

Malignant gliomas are the most common type of primary brain tumor. They are classified on the basis of the World Health Organization (WHO) grading system. Pathological diagnosis helps ascertain the biology and behavior of brain tumors. The most commonly used consensus approach for the diagnosis of malignant gliomas is to classify the tumors as astrocytic tumors, that is, anaplastic astrocytoma (AA), glioblastoma (GBM),anaplastic oligodendroglioma (AO), and anaplastic oligoastrocytoma (AOA). An accurate distinction between the different types of malignant gliomas is important for deciding the prognosis and therapeutic approaches. Thus far, histopathological examination is the gold standard for the typing and grading of gliomas. However, this method is associated with significant inter-observer variability. Furthermore, the clinical behavior of individual tumors having specific pathology might differ substantially. Thus, additional markers are needed for refined and more objective glioma classification, better prediction of prognosis, and tailored therapeutic decision-making. At present, clinical factors such as age, Karnofsky performance status (KPS), and resection rate are primarily used to predict the prognosis.

Unlike the classical molecular markers for gliomas - *p53* and epidermal growth factor receptor (*EGFR*) status - the clinical significance of which has remained controversial, at least three important molecular markers with clinical implications have now been identified. These are 1p/19q co-deletion, O^6-methylguanine methyltransferase (*MGMT*) promoter methylation, and isocitrate dehydrogenase-1 (*IDH1*) mutations.

Chromosome 1p/19q co-deletion was first reported in oligodendroglial tumors in 1994 [1]. Cairncross *et al.* reported chemosensitivity in patients with AOs harboring deletion of 1p, particularly co-deletion of 1p and 19q [2]. Almost 85% of low-grade oligodendrogliomas and 65% of AOs harbor 1p/19q co-deletion [3]. The potential role of 1p/19q loss in therapeutic decision-making in AOs has been analyzed in large studies. The 1p/19q deletions were incorporated into three major therapeutic trials in patients with AO. All the trials confirmed the prognostic and possible predictive role of this biomarker at initial therapy [4-6].

MGMT promoter methylation is the only potentially predictive marker, especially for alkylating agent chemotherapy in glioblastoma. At present, temozolomide (TMZ) is mainly used for the treatment of malignant gliomas [7], and many clinical studies on TMZ have been performed. TMZ is a DNA-methylating agent and exerts its cytotoxicity by adding a methyl group to the O^6 position of guanine residues on DNA. This induces DNA mismatch, DNA double-stand breaks, and apoptosis in proliferating cells [8]. *MGMT*, a DNA repair enzyme, is known to induce resistance to chemotherapy in some patients with malignant gliomas. In a tumor with a hypermethylated *MGMT* promoter, *MGMT* expression is reduced and cytotoxicity of alkylating agents is enhanced. Stupp *et al.* suggested that the combination of TMZ with radiotherapy could be used as the initial standard treatment for GBM [9]; they also investigated whether the state of *MGMT* activity could be a prognostic factor. Cancer-specific DNA methylation changes are hallmarks of human cancers, with global DNA hypomethylation often seen concomitantly with hypermethylation of CpG islands [10]. A CpG island methylator phenotype (CIMP) is regarded as cancer-specific CpG island hypermethylation of a subset of genes in some tumors [11]. In GBM, glioma-CIMP status (G-CIMP) has been shown to be a significant predictor of improved patient survival [12]. Collectively, these different sets of observations suggest that the level of *MGMT* promoter methylation, serving as a prognostic factor, may reflect an aspect of the global DNA methylation status in GBM.

In 2008, Volgelstein *et al.* conducted a comprehensive sequence analysis in 22 patients with GBM and identified *IDH1* mutation as a new driver mutation [13]. In another analysis, they detected *IDH1* mutations in 18 (12%) of 149 patients with GBM. Clinically, patients with *IDH1* mutations are characterized by the occurrence of secondary GBM and early disease onset [14,15]. A large-scale study revealed *IDH1* mutations in 50% to 80% of patients with grade 2 astrocytoma, oligodendroglioma, or secondary GBM; however, *IDH1* mutations were rare in patients with primary GBM [6,16-24]. Thus, *IDH1* mutations may be considered new molecular diagnostic markers. In addition, recent studies showed that patients with *IDH1* mutations had a better outcome than those with wild-type *IDH1* [6,16-24]. The biological function of *IDH1* mutations has not yet been completely understood. Wild-type *IDH1* oxidizes isocitrate to α-ketoglutarate (α-KG) and reduces nicotinamide adenine dinucleotide phosphate (NADP) to NAPD-oxidase (NADPH) [25]. Mutated *IDH1* reduces the activity of NADPH, which is required for cellular defense against oxidative stress, leading to tumorigenesis because of oxidative DNA damage [26]. Furthermore, this mutation results in a new function of *IDH1* leading to the conversion of α-KG to 2-hydroxyglutarate (2HG), which promotes the accumulation of hypoxia-inducible factor (HIF)1α, leading to vascular endothelial growth factor signaling-mediated tumorigenesis *in vitro* [27]. However, Metellus *et al.* question the actual relationship between *IDH* mutation status and *in vivo* hypoxic biomarkers [28]. Also Chowdhury *et al.* showed that 2HG inhibits 2-oxoglutarate (2OG)-dependent oxygenases with varying potencies and indicated that candidate oncogenic pathways in *IDH*-associated malignancy should include those that are regulated by other 2OG oxygenases than HIF hydroxylases [29]. Despite its obvious association with

tumorigenesis, the relationship between *IDH1* mutation and good prognosis for malignant glioma is yet unknown.

We evaluated the significance of these markers, that is, 1p/19q co-deletion, *MGMT* promoter methylation, and *IDH1* mutations, in malignant glioma. The objective of the present study was to confirm the difference in the prognostic impacts of *MGMT* methylation status and *IDH1* mutation and 1p/19q co-deletion in patients with GBM and AA and those with AO and AOA, respectively.

Methods

In this study, patients with malignant glioma were divided into two groups according to the presence of the oligodendroglioma component. Groups 1 and 2 consisted of patients with GBM and AA and those with AO and AOA, respectively.

Patient and tissue specimens

Between 1996 and 2009, 267 patients with malignant glioma (30 with AO, 26 with AOA, 40 with AA, 159 with primary GBM and 12 with secondary GBM) treated at Kumamoto University Hospital were included in this study. Tumor specimens were obtained by surgical resection (including biopsy), quick-frozen in liquid nitrogen, and maintained at -80°C until use. The patients and/or their legal guardians provided written informed consent for use of the specimens. Formalin-fixed, paraffin-embedded specimens were pathologically examined. Each specimen was classified by the local neuropathologists according to the WHO criteria. The tumor type *IDH1* mutational status, *MGMT* methylation status, age and gender distribution, Karnofsky performance status (KPS) score, and median survival time are shown in Table 1.

Direct DNA sequencing of *IDH1* mutations

Genomic DNA was isolated from the surgical specimens using the Qiagen kit (Qiagen, Valencia, CA, USA). The PCR primers for genomic region corresponding to *IDH1* exon 4 that encodes codon R132 were as follows: *IDH1* sense (5'-AAACAAATGTGGAAATCACC-3') and *IDH1* antisense (5'-TGCCAACATGACTTACTTGA-3'). The PCR conditions were 94° for 5 minutes; 36 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 minute; and extension at 72°C for 5 minutes. The PCR was performed using Ex-Taq HS DNA Polymerase (Takara Bio, Shiga, Japan). The PCR products were purified using QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. Sequencing reactions were performed using previous primers and a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Life Technologies, Carsbad, CA, USA) on an ABI377 automated sequencer (Applied Biosystems).

Table 1 Patients and characteristics

Characteristic	Histologic subtype			
	AO (n = 30)	AOA (n = 26)	AA (n = 40)	GBM (n = 171)
Gender				
Male/female ratio	0.76	1.36	1.22	1.59
Male, n	13	15	22	105
Female, n	17	11	18	66
Age, years				
Median	45.0	49.5	45.5	61.0
Range	16 to 77	30 to 65	10 to 72	3 to 81
Karnofsky performance status				
Median	100	100	90	90
Range	40 to 100	70 to 100	40 to 100	20 to 100
Surgery				
Total removal, n	22	13	8	74
Partial removal, n	7	12	21	73
Biopsy, n	1	1	11	24
***IDH1* mutation, n**	20(66.7%)	12(46.2%)	12(30.0%)	12(7.0%)
***MGMT* promoter methylation, n**	24(80.0%)	19(73.1%)	18(45.0%)	73(42.7%)
1p/19q co-deletion, n	18(60.0%)	11(42.3%)		
Survival, months, median	70.5	80.0	40.0	14.0

AO, anaplastic oligodendroglioma; AOA, anaplastic oligoastrocytoma; AA, anaplastic astrocytoma; GBM, glioblastoma; n, number of patients.

Methylation-specific PCR for *MGMT* promoter

MGMT methylation was detected using methylation-specific PCR (MSP). Genomic DNA from each sample (2 μg) was treated with sodium bisulfite using the Epitect Bisulfite Kit (Qiagen Valencia, CA). The primer sequences for the unmethylated reaction were 5'-TTTGTGTTTT GATGTTTGTAGGTTTTTGT-3' (forward) and 5'-AACT CCACACTCTTCCAAAAACAAAACA-3' (reverse), and those for the methylated reaction were 5'-TTTCGAC GTTCGTAGGTTTTCGC-3' (forward) and 5'-GCACTC TTCCGAAAACGAAACG-3' (reverse). The PCR conditions were as follows: 95° for 5 minutes; 34 cycles of 95° for 30 s, 61° for 30 s, 72° for 30 s; and extension at 72° for 4 minutes. Amplified products were separated on 3% agarose gels, stained with ethidium bromide, and visualized under UV illumination.

1p/19q co-deletion analysis by fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization (FISH) was performed according to the method described previously [30]. Control and detecting probes were developed from plasmids D1Z1 (1q12) and D1Z2 (1p36.3) for the chromosome 1 study and from bacterial artificial chromosomes (BACs)

Table 2 Clinical and genetic features of patients with malignant glioma with and without isocitrate dehydrogenase 1 (*IDH1*) mutation

		IDH1		
		Mutation (+)	Mutation (-)	*P*-value
AO	**Cases, number**	20	10	
	Gender			
	Male, number	8	5	NS
	Female, number	12	5	
	Age, mean, years	48.3	44.4	NS
	Karnofsky performance status, mean score, %	94.5	89	NS
	Surgery			
	Total, number	16	6	NS
	Partial or biopsy, number	4	4	
	***MGMT* promoter**			
	Methylation (+), number	19	5	0.0155
	Methylation (-), number	1	5	
	1p 19 co-deletion, number	11	7	NS
	Survival, median, months	72	69	NS
AOA	**Cases, number**	12	14	
	Gender			
	Male, number	5	10	NS
	Female, number	7	4	
	Age, mean, years	46.4	48.7	NS
	Karnofsky performance status, mean score, %	97.5	96.4	NS
	Surgery			
	Total, number	5	8	NS
	***MGMT* promoter**			
	Methylation (+), number	11	8	0.0479
	Methylation (-), number	1	6	
	1p 19q co-deletion, number	7	4	NS
	Survival, median, months	88	65	NS
AA	**Cases, number**	12	28	
	Gender			
	Male, number	8	14	NS
	Female, number	4	14	
	Age,mean, years	41.7	44.3	NS
	Karnofsky performance status, mean score, %	90.8	78.9	NS
	Surgery			
	Total, number	4	4	NS
	Partial or biopsy, number	8	24	
	***MGMT* promoter**			
	Methylation (+), number	9	9	0.0125
	Methylation (-), number	3	19	
	Survival, median, months	55	25	0.0786

Table 2 Clinical and genetic features of patients with malignant glioma with and without isocitrate dehydrogenase 1 (*IDH1*) mutation *(Continued)*

GBM	**Cases, number**	12	159	
	Tumor occurrence			
	Primary, number	4	155	0.0001
	Secondary, number	8	4	
	Gender			
	Male, number	6	99	NS
	Female, number	6	60	
	Age, mean, years	43.8	58.5	0.004
	Karnofsky performance status, mean score, %	87.5	79.7	NS
	Surgery			
	Total, number	3	71	NS
	Partial or biopsy, number	9	88	
	***MGMT* promoter**			
	Methylation (+), number	10	63	0.0032
	Methylation (-), number	2	96	
	Survival, median, months	20	14	0.0051

RP11-413 M18 (19q13) and CTZ-2571 L23 (19q13.3) for chromosome 19 study, respectively. Dual-colored probes against chromosomes 1p and 19q were used to detect chromosomal loss at these loci - a single fluorescent signal in the nucleus was interpreted as chromosomal-arm loss if two signals were detected for the control probe.

Statistical analyses

The Student *t*-test was used to compare the mean age and KPS of patients with *IDH1* mutations. The Chi-square test was used to analyze the significance of the association between *IDH1* mutation and the following data: gender, resection rate, and *MGMT* methylation status. The overall survival was defined as the time between the first surgery and death. Survival distributions were estimated by Kaplan-Meier analysis and statistically analyzed using the log-rank test. Univariate and multivariate analysis was performed using the Cox, nonparametric proportional hazards regression model to estimate the relative risk (RR) for age, extent of resection, *IDH1* mutation status, *MGMT* status and diagnosis in group 1 and for age, extent of resection, *IDH1* mutation status, *MGMT* status, existence of 1p19q co-deletion and diagnosis in group 2, respectively. All statistical analyses were performed using StatView 5.0 (SAS Institute Inc., Cary, NC, USA).

Results

IDH1 mutations in malignant gliomas

The 56 mutations of *IDH1* genes were identified in all malignant gliomas (21.1%) of the R132H type. Patients with *IDH1* mutations were significantly younger than those without IDH1 mutations (mean age, 45.5 versus 55.5 years, $P < 0.0001$). The difference in mean age was more evident in patients with GBM who had *IDH1* mutations than in those without (mean age, 43.8 versus 58.5 years, $P = 0.004$) (Table 2). *IDH1* mutations were predominantly observed in the patients with secondary GBM (8 of 12, 66.7%) but rarely in patients with primary GBM (4 of 159, $P < 0.0001$) (Table 2).

MGMT promoter methylation and 1p/19q co-deletion in malignant gliomas

Of the 267 malignant glioma patients, 134 exhibited *MGMT* promoter methylation (49.4%). *MGMT* promoter methylation was considerably higher in patients with AO and AOA (80.0% and 73.1%, respectively), but relatively lower in patients with GBM (42.7%) (Table 1). Combined 1p/19q loss of heterozygosity (LOH) was noted in 60.0% AO and 42.3% AOA patients (Table 1).

Correlation of *IDH1* mutations with *MGMT* promoter methylation and 1p/19q LOH

Gene sequence analysis showed a significant correlation of *IDH1* mutations with *MGMT* gene promoter methylation ($P < 0.0001$). *MGMT* methylation was noted in 83.3%, 75.0%, 91.7%, and 95.0% of patients with GBM, AA, AOA, and AO who had *IDH1* mutations, respectively. However, there was no significant correlation between *IDH1* mutations and LOH status of 1p/19q (Table 2).

Survival of patients according to *IDH1* status

In group 1, patients with *IDH1* mutations had significantly longer survival time than those with wild-type *IDH1* (Figure 1a). In group 2, the survival time of patients with

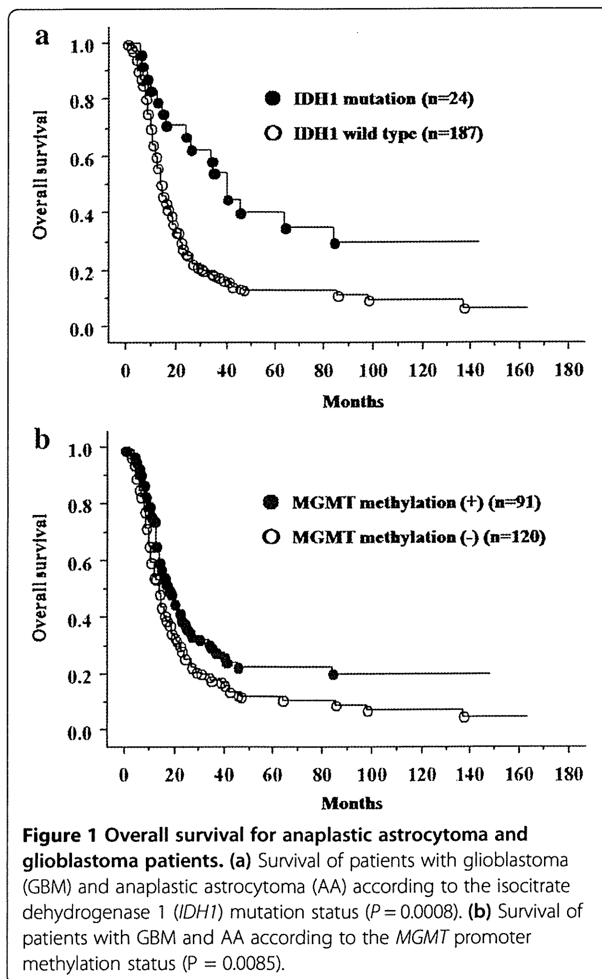

Figure 1 Overall survival for anaplastic astrocytoma and glioblastoma patients. **(a)** Survival of patients with glioblastoma (GBM) and anaplastic astrocytoma (AA) according to the isocitrate dehydrogenase 1 (*IDH1*) mutation status ($P = 0.0008$). **(b)** Survival of patients with GBM and AA according to the *MGMT* promoter methylation status ($P = 0.0085$).

IDH1 mutations was slightly longer than that of patients without *IDH1* mutations (Figure 2a).

Survival of patients according to *MGMT* methylation status and 1p/19q co-deletion

For groups 1 and 2, patients with *MGMT* methylation had a longer survival time than those without (Figure 1b and Figure 2b). In group 2, patients with 1p/19q co-deletion had significantly better outcome than those without (Figure 2c).

Univariate and multivariate analysis

Table 3 summarizes the significant variables. Univariately, age, gender, *IDH1* status, MGMT methylation status and histology were positively correlated with increased overall survival in group 1 (AA + GBM) ($P < 0.05$). In multivariate analysis, age, resection rate, *MGMT* status and histology were independent prognostic factor for improved overall survival in group 1 ($P < 0.05$). Also, univariate analysis

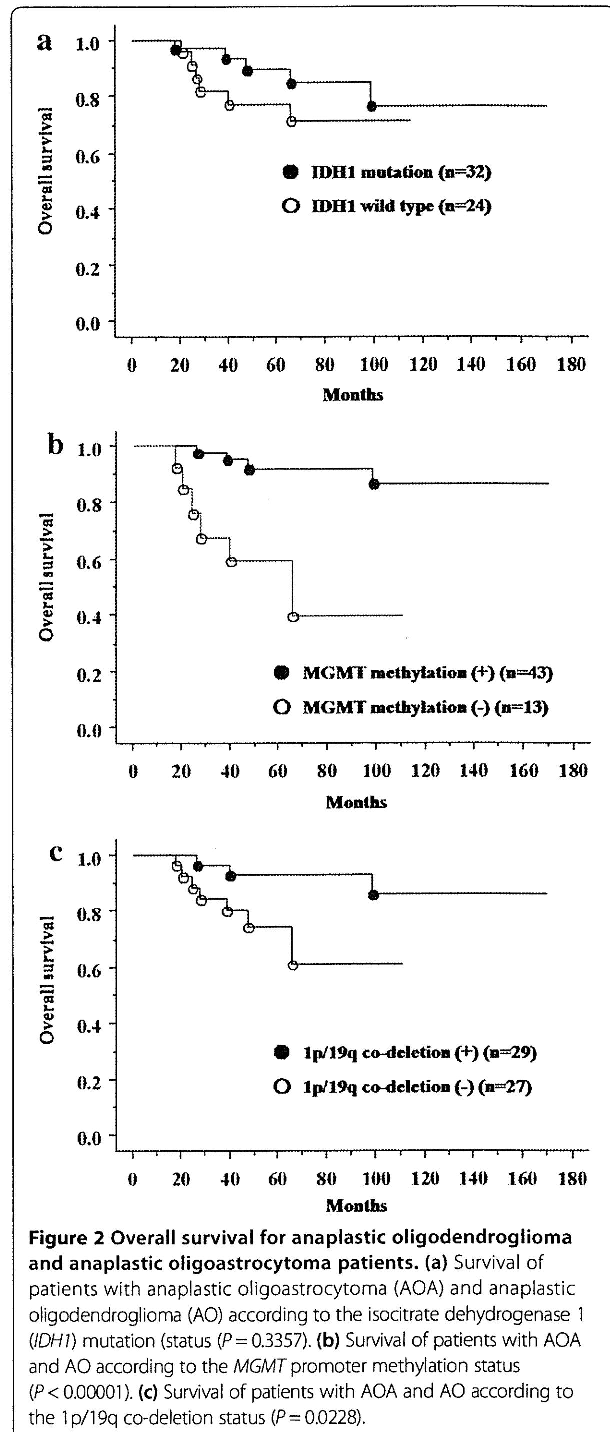

Figure 2 Overall survival for anaplastic oligodendroglioma and anaplastic oligoastrocytoma patients. **(a)** Survival of patients with anaplastic oligoastrocytoma (AOA) and anaplastic oligodendroglioma (AO) according to the isocitrate dehydrogenase 1 (*IDH1*) mutation (status ($P = 0.3357$). **(b)** Survival of patients with AOA and AO according to the *MGMT* promoter methylation status ($P < 0.00001$). **(c)** Survival of patients with AOA and AO according to the 1p/19q co-deletion status ($P = 0.0228$).

showed that overall survival was significantly impacted by resection rate, *MGMT* methylation status and existence of 1p19q co-deletion in group 2 (AO + AOA) ($P < 0.05$). In multivariate analysis, age, gender and *MGMT* status were found to be independently associated with improved overall survival in group 2 ($P < 0.05$).

Table 3 Univariate and multivariate analysis of factors associated with survival

	Univariate Cox regression			Multivariate Cox regression		
	HR	95% CI	P-value	HR	95% CI	P-value
Group 1 (AA + GBM)						
Age (per year)	1.023	1.014–1.033	<0.0001	1.023	1.013–1.034	<0.0001
Gender (female versus male)	1.023	1.014–1.033	<0.0001	0.810	0.590–1.112	0.1928
Resection (total resection versus non-total resection)	1.348	0.987–1.840	0.06	1.994	1.440–2.763	<0.0001
IDH1 (mutation versus wild-type)	0.427	0.253–0.719	0.0014	0.708	0.403–1.243	0.2290
MGMT (methylation versus unmethylation)	0.671	0.494–0.911	0.0106	0.614	0.442–0.852	0.0035
Histology (AA versus GBM)	0.372	0.242–0.571	<0.0001	0.419	0.264–0.666	0.0002
Group 2 (AO + AOA)						
Age (per year)	1.025	0.971–1.083	0.3672	1.094	1.003–1.193	0.0421
Gender (female versus male)	0.499	0.145–1.717	0.2703	0.156	0.027–0.890	0.0365
Resection (total resection versus non-total resection)	0.886	0.289–3.031	0.037	0.852	0.178–4.074	0.8412
IDH1 (mutation versus wild-type)	0.563	0.172–1.848	0.3436	2.271	0.415–12.444	0.3444
MGMT (methylation versus unmethylation)	0.115	0.033–0.402	0.0007	0.041	0.007–0.257	0.0006
1p19q (co-deletion versus non co-deletion)	4.208	1.099–16.114	0.0359	4.720	0.685–32.526	0.1150
Histology (AO versus AOA)	0.723	0.220–2.377	0.5937	1.935	0.383–9.785	0.4247

AO, anaplastic oligodendroglioma; AOA, anaplastic oligoastrocytoma; AA, anaplastic astrocytoma; GBM, glioblastoma; HR, hazard ratio.

Discussion

Recently, molecular markers have been increasingly used for the assessment and management of malignant glioma. Some molecular signatures are used diagnostically to help pathologists classify tumors, whereas others are used to estimate the prognosis for patients. In this study, we focused on 1p/19 co-deletion, *MGMT* promoter methylation status, and *IDH1* mutations in patients with malignant glioma.

Genetic mutations are classified into two types: driver mutations, which are involved in causing and promoting cancer, and passenger mutations, which occur concomitantly as a result of driver mutations. *IDH1* mutations have been identified as a new driver mutation by a comprehensive sequence analysis in 22 patients with GBM [13]. Interestingly, these *IDH1* mutations were associated with young patient age and secondary GBMs. This observation drew attention to diffuse astrocytoma and AA, both of which were found to carry *IDH1* mutations in the majority of cases [6,16-24]. As expected, our study also showed high frequency of *IDH1* mutations in patients with secondary GBM (66.7%) and grade 3 glioma (for example, 12 (30.0%) of 40 patients with AA, 12 (46.2%) of 26 patients with AOA, and 20 (66.7%) of 30 patients with AO), whereas the frequency was lower in patients with primary GBM (2.6%). Thus, *IDH1* mutations are thought to play an important role in the early phase of glioma development.

A relationship between good prognosis and presence of *IDH1* mutations was reported by analyzing patients with GBMs [24], AAs [6], and AOs [22]. Thus, in addition to the conventional pathological diagnosis, classification of patients on the basis of the presence or absence of *IDH1* mutations should be considered for patients with malignant glioma (GBM and AA). A study suggested that the presence of an *IDH1* mutation is a prognostic factor in AO patients [22]; however, our present study showed only slight improvement in survival of AO and AOA patients with *IDH1* mutations. Despite the absence of *IDH1* mutations, our group-2 patients had a good prognosis. In a group that includes many long survivors, determining the prognostic value becomes difficult. The difference in our results and the previous findings may be due to this reason.

MGMT promoter methylation has been identified in a wide range of human cancers [31]. Promoter methylation was responsible for the inactivation of this gene. *MGMT* methylation has been reported in 35% to 73% of patients with GBM [7,8,24,32-42] and 50% to 84% of patients with grade3 glioma [6,41,43]. The reported frequencies varied across studies because of the different analysis methods and conditions used in these studies. Our MS-PCR analysis showed the following frequencies of *MGMT* methylation: 42.7% (73/171), 45.0% (18/40), 73.1% (19/26), and 80.0% (24/30) for GBM, AA, AOA, and AO patients, respectively. Our study also showed significantly greater *MGMT* methylation in malignant glioma patients with *IDH1* mutations than in those without ($P < 0.0001$). Thus, these two genetic changes might have some relationship. Depending on the primers used and MS-PCR conditions, the obtained results may differ across different studies.

All *IDH1* mutations in our study involved the 132G395A mutant. G-to-A mutations are commonly found in *TP53* and *K-Ras* genes in patients with *MGMT* methylation

[8,44]. Such common G-to-A mutations may account for the higher frequency of 132G395A mutations in the *IDH1* codon in patients with *MGMT* methylation.

Loss of 1p and 19q is thought to be the genetic hallmark of oligodendroglial tumors. The frequency of 1p/19q co-deletion was 60.0% in AO and 42.3% in AOA patients. Many studies, including three prospective randomized phase III trials, suggested that 1p/19q deletion was a powerful prognostic marker in patients with WHO grade-3 gliomas. Importantly, these studies also indicated that the prognostic power was independent of the type of adjuvant therapy, that is, radiotherapy, chemotherapy, or combined radiotherapy/chemotherapy [4-6]. We also found significantly better outcomes in Japanese patients with 1p/19q co-deletion.

Regardless of the histological diagnosis made on the basis of the WHO classification, the surgical resection rate is considered an important prognostic factor [45,46]. Thus, we investigated the relationship between the surgical resection rate and genetic changes in *IDH1* or *MGMT* in GBM and AA patients. We obtained pre- and post-contrast magnetic resonance imaging (MRI) less than 72 hours after surgery in every case and pre-contrast and post-contrast images were compared. Enhanced areas were considered to be tumors except for obvious vessel images. The resection rate was calculated as percent change of residual tumor over preoperative T1 gadolinium (Gd) volume in all cases (100%, total removal; 95% to 5%, partial removal; below5%, biopsy). We intended to maximum resection without causing neurological morbidity. Depending on the surgical resection rate, group 1 patients were further divided into the following two subgroups: those in whom total resection was successful and those in whom total resection was not possible. In patients with *IDH1* mutations in whom total resection was not performed, the survival curves were very similar to those of patients with wild-type *IDH1* in whom total resection was performed (Figure 3). Despite the small sample size, our study suggested that the survival time of patients with *IDH1* mutations who undergo total resection is longer. If any *IDH1* mutation is considered as a marker, surgeons would be able to change their treatment strategies, including the choice of surgical procedures. Furthermore, irrespective of the *MGMT* methylation status, the prognosis of glioma patients can be improved if total resection is performed.

These findings suggest that molecular biological analyses can be used to predict the prognosis of each patient. Thus, besides the pathological diagnosis made on the basis of the existing classification system alone, developing a new classification system assessing genetic changes, such as *IDH1* mutations and the status of *MGMT* methylation and 1p/19q co-deletion, is necessary. This new classification system will allow the design of novel treatment strategies. However, information on these three genetic changes might not always be necessary. GBA and AA patients with *IDH1* mutations and *MGMT* methylation had longer survival times than those without such genetic changes. The tendency for longer survival was more marked in the subgroup with *IDH1* mutations than in those with *MGMT* methylation. Hence, for GBM or AA patients, a classification made on the basis of the presence or absence of *IDH1* mutations seems reasonable; however, that made on the basis of the *MGMT* methylation status should be discussed more carefully. The difference in the degree of association of *IDH1* mutations with prognostic factors between group 1 (GBM + AA) and group 2 (AO + AOA) patients was not clear. This could be because different numbers of patients were included in the groups. Therefore, further analyses involving a greater number of patients are necessary.

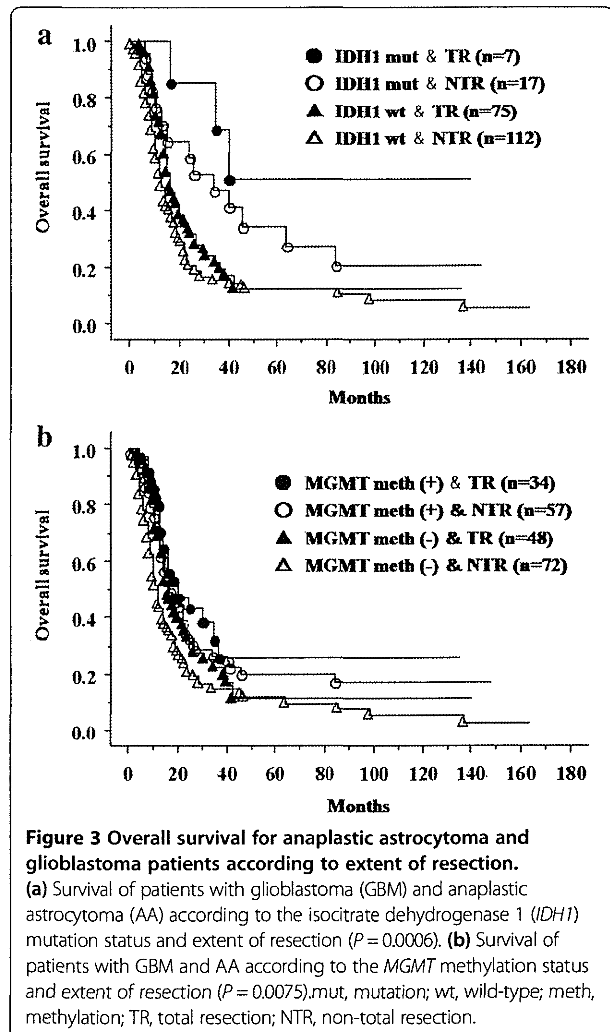

Figure 3 Overall survival for anaplastic astrocytoma and glioblastoma patients according to extent of resection. **(a)** Survival of patients with glioblastoma (GBM) and anaplastic astrocytoma (AA) according to the isocitrate dehydrogenase 1 (*IDH1*) mutation status and extent of resection ($P = 0.0006$). **(b)** Survival of patients with GBM and AA according to the *MGMT* methylation status and extent of resection ($P = 0.0075$).mut, mutation; wt, wild-type; meth, methylation; TR, total resection; NTR, non-total resection.

Similarly, AOA and AO patients should be evaluated by taking into account the status of *MGMT* methylation and 1p/19q co-deletion, and not the *IDH1* mutation status.

Conclusions

In summary, our study adds further support for the significant roles of *IDH1* mutations and *MGMT* methylation in the prognosis of GBM and AA patients and suggests that *IDH1* mutations might serve as a more potent prognostic factor. In contrast, *MGMT* methylation and 1p/19q co-deletion status, rather than *IDH1* mutation status, were prognostic factors in Japanese patients with AOA and AO. Furthermore, our study highlighted the importance of total resection in GBM and AA patients with *IDH1* mutations. Moreover, our study includes the largest number of Japanese patients with malignant gliomas that has been analyzed for these three markers. We believe that our findings will increase the awareness of oncologists in Japan of the value of these markers for predicting prognosis and designing appropriate therapeutic strategies for treating this highly fatal disease.

Abbreviations

2HG: 2-hydroxyglutarate; 2OG: 2-oxoglutarate; AA: anaplastic astrocytoma; α-KG: α-ketoglutarate; AO: anaplastic oligodendroglioma; AOA: anaplastic oligoastrocytoma; CIMP: CpG island methylator phenotype; EGFR: epidermal growth factor receptor; FISH: fluorescence *in situ* hybridization; GBM: glioblastoma; G-CIMP: glioma-CpG island methylator phenotype; HIF: hypoxia-inducible factor; HR: hazard ratio; IDH1: isocitrate dehydrogenase 1; KPS: Karnofsky performance status; LOH: loss of heterozygosity; MGMT: O^6-methylguanine-DNA methyltransferase; MSP: methylation-specific polymerase chain reaction; NAPD: nicotinamide adenine dinucleotide phosphate; NAPDH: nicotinamide adenine dinucleotide phosphate-oxidase; PCR: polymerase chain reaction; RR: relative risk; TMZ: temozolomide; WHO: World Health Organization.

Competing interests

None of the authors have any financial support or conflicts of interest associated with this study.

Authors' contributions

YT performed all the experiments and drafted the manuscript. HN was involved in the final version of the manuscript. KM, TH and DM participated in the analyses of FISH and methylation specific PCR. HK and JK oversaw the design of the study. All authors have read and approve the final version of the manuscript.

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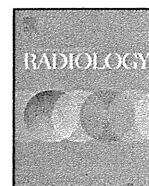

Advantages of high *b*-value diffusion-weighted imaging to diagnose pseudo-responses in patients with recurrent glioma after bevacizumab treatment

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ABSTRACT

Background: The diagnosis of pseudo-responses after bevacizumab treatment is difficult. Because diffusion-weighted imaging (DWI) is associated with cell density, it may facilitate the differentiation between true- and pseudo-responses. Furthermore, as high *b*-value DWI is even more sensitive to diffusion, it has been reported to be diagnostically useful in various clinical settings.
Materials and methods: Between September 2008 and May 2011, 10 patients (5 males, 5 females; age range 6–65 years) with recurrent glioma were treated with bevacizumab. All underwent pre- and post-treatment MRI including T2- or FLAIR imaging, post-gadolinium contrast T1-weighted imaging, and DWI with *b*-1000 and *b*-4000. Response rates were evaluated by MacDonald- and by response assessment in neuro-oncology working group (RANO) criteria. We also assessed the response rate by calculating the size of high intensity areas using high *b*-value diffusion-weighted criteria. Prognostic factors were evaluated using Kaplan–Meier survival curves (log-rank test).
Results: It was easier to identify pseudo-responses with RANO- than MacDonald criteria, however the reduction of edema by bevacizumab rendered the early diagnosis of tumor progression difficult by RANO criteria. In some patients with recurrent glioma treated with bevacizumab, high *b*-value diffusion-weighted criteria did, while MacDonald- and RANO criteria did not identify pseudo-responses at an early point after the start of therapy.
Discussion and conclusion: High *b*-value DWI reflects cell density more accurately than regular *b*-value DWI. Our findings suggest that in patients with recurrent glioma, high *b*-value diffusion-weighted criteria are useful for the differentiation between pseudo- and true responses to treatment with bevacizumab.

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1. Introduction

Glioblastoma is the most common malignant primary neoplasm of the central nervous system. Despite aggressive treatment, it almost always recurs with fatal consequences. As vascular endothelial growth factor (VEGF) and its receptors are highly expressed in glioblastoma, VEGF may constitute an important molecular target in its treatment. VEGF increases vascular permeability and contributes to contrast enhancement and the peritumoral edema associated with these tumors. Anti-angiogenic agents, especially those targeting VEGF such as bevacizumab, can significantly reduce vascular permeability. This results in diminution of the enhanced lesion irrespective of changes in the tumor size. Therefore, it is very difficult to determine the responder status of glioma patients treated with bevacizumab on conventional MR images and some tumors thought to have responded to bevacizumab therapy exhibit progression without manifesting an increase in the size of the gadolinium-enhanced tumor. This phenomenon, defined as a "pseudo-response", has been observed immediately after the start of treatment and renders the accurate assessment of a true tumor response difficult [1–3]. Emerging evidence of survival prolongation in patients who responded to bevacizumab [4] suggests that it exerts antitumor effects. Reliable means to assess the treatment response and the progression of these tumors addressed with anti-angiogenic agents must be developed.

The response assessment based on neuro-oncology working group (RANO) criteria takes into account increases in the enhanced tumor size, the T2/FLAIR high size, the dose of corticosteroids, and clinical symptoms. Using RANO criteria, it may be possible to identify tumor progression after treatment with bevacizumab because post-treatment the non-enhanced tumor area tends to increase without an increase in the size of the enhanced tumor. This may

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also be reflected by an increase in the size of the T2/FLAIR high-intense lesion. On the other hand, as treatment with bevacizumab may reduce the size of brain edema, it may be difficult to distinguish between true- and pseudo-response at an early point after treatment with bevacizumab.

As the apparent diffusion coefficient (ADC) calculated from diffusion-weighted (DW) images is associated with tumor cellularity [5], it is considered an important biomarker of cancer [6,7]. The ADC has also been used to assess the response of brain tumors to therapy [7] and to predict survival in patients with newly diagnosed glioblastoma [8]. DWI studies at higher diffusion gradient strength (*b*-values) have been used for the diagnosis of acute stroke [9], the assessment of lesion-to-normal contrast in neurodegenerative diseases [10], the prediction of the glioma grade [11], and for the differentiation between glioblastoma and malignant lymphoma [12]. The aim of this study was to evaluate whether RANO criteria and DW imaging including high *b*-value DW (HBDW) imaging could assess the pseudo-response after treatment with bevacizumab. Here we show that HBDW imaging may represent a useful tool for the diagnosis of pseudo-responses in glioblastoma patients treated with bevacizumab.

2. Materials and methods

2.1. Patients and MR imaging

Between September 2008 and May 2011, 10 patients (5 males, 5 females; age range 6–65 years) with recurrent glioma were treated with bevacizumab in our institutions. Recurrence before the administration of bevacizumab was defined by MacDonald criteria [13].

All MRI studies were performed on a 3 T superconducting system (Signa Excite HD 3.0T; GE Medical Systems, Milwaukee, WI, USA). All patients underwent pre- and post-treatment magnetic resonance (MR) imaging including T2- (TR 4800 ms, TE 100 ms, echo train length 18, field-of-view (FOV) 22 cm × 22 cm, matrix size 512 × 320/2NEX, section thickness 6 mm, intersection gap 1.0 mm, 1 acquisition) or FLAIR imaging (TR 10,000 ms, TE 140.0 ms, inversion recovery time 2400.0 ms, FOV 22 cm × 22 cm, matrix size 288 × 160/1NEX, section thickness 6 mm, intersection gap 1.0 mm, 2 acquisitions), gadolinium-enhanced T1-weighted imaging (TR 450 ms, TE 18 ms, FOV 22 cm × 22 cm, matrix size 256 × 192/1NEX, section thickness 6 mm, intersection gap 1.0 mm, 2 acquisitions), and DW imaging at $b = 1000$ and $b = 4000$ s/mm. The parameters at *b*-1000 and *b*-4000 DWI were: 8-channel phased array head coil, TR 5000 ms, TE 66.2 ms (*b*-1000) and TR 5000 ms and TE 96.4 ms (*b*-4000), NEX 1, FOV 220, slice thickness 6 mm, gap 1.0 mm, number of slices 20, data acquisition matrix 128 × 128, scan time 20 s (*b*-1000) and 40 s (*b*-4000).

2.2. Response after treatment with bevacizumab

The response rate was determined using 3 different methods. Under MacDonald criteria [13], the enhanced tumor size was calculated and defined as complete response (CR = disappearance of all enhanced target lesions), partial response (PR = a 50% decrease from the baseline), stable disease (SD = neither PR- nor progressive disease (PD) criteria are met), PD (a 25% increase over the smallest sum recorded or the appearance of new lesions), the clinical assessment and corticosteroid dose were also recorded. Under the criteria of the response assessment in neuro-oncology (RANO) working group [2], factors such as enhanced tumor size, T2/FLAIR high size, dose of corticosteroids, and clinical symptoms were taken into account. At visual inspection, HBDW (*b*-4000) imaging was superior to regular *b*-value based (*b*-1000) DW imaging for the assessment of size changes of high-intense lesions. Therefore, under the third method we calculated the size of the high-intense lesion on HBDW images using its two dimensional measurements and established HBDW criteria where CR = disappearance of all high intensity lesions on HBDW images, PR = a 50% decrease from the baseline of high intensity lesions observed on HBDW images, SD = neither PR nor PD criteria are met, PD = a 25% increase over the smallest sum recorded or the appearance of new DW high lesions on HBDW images.

2.3. Statistical analysis

Statistical analyses were with PRISM version 5.0 (GraphPad Software Inc., La Jolla, CA, USA). The survival time of patients with recurrent glioma was measured from the time of initial treatment with bevacizumab to the time of death or last follow-up. To evaluate prognostic values we performed Kaplan–Meier survival analysis (log-rank test) that incorporated the response to bevacizumab based on MacDonald-, RANO-, or HBDW criteria.

3. Results

Table 1 presents a summary of our patients. Their age ranged from 6 to 65 years (mean 42.5 years, median 40 years). Based on MacDonald criteria, the initial response rate was CR, $n = 4$; PR, $n = 4$; SD, $n = 1$; PD, $n = 1$; under RANO criteria it was CR, $n = 2$; PR, $n = 3$; SD, $n = 3$; PD, $n = 2$, and under HBDW criteria, the initial response rate was PR, $n = 3$; SD, $n = 3$; PD, $n = 4$ patients.

After bevacizumab administration, the enhanced lesion disappeared in 5 tumors and based on MacDonald criteria CR was recorded. In 3 patients there was a decrease in the size of both the T2/FLAIR- and the HBDW high intense lesion; based on RANO and HBDW criteria, PR was recorded (Fig. 1, case 5). These patients are currently alive without recurrence and their treatment with bevacizumab continues.

In some patients the high intensity lesion on T2/FLAIR- and HBDW images increased (Fig. 2, case 1). They were categorized as PD under RANO or HBDW criteria. After continued treatment with bevacizumab, they were recorded as PD. In some patients there was a decrease in the size of the T2/FLAIR high intense lesion after one cycle of bevacizumab. However, in 2 patients the high intense lesion became larger on HBDW images (Fig. 3, case 2) and based on RANO criteria PD was recorded after the continuation of bevacizumab treatment.

We performed Kaplan–Meier survival analysis based on the response rate determined by MacDonald-, RANO-, and HBDW criteria. Under MacDonald criteria we observed no statistical difference between CR/PR- and SD/PD patients (Fig. 4A). Under RANO criteria there was a statistical difference between CR/PR- and SD/PD patients ($p = 0.0153$, Fig. 4B) and under HBDW criteria the difference was more obvious and CR/PR patients survived longer ($p = 0.0152$, Fig. 4C).

4. Discussion

Our study documents that in patients with recurrent glioblastoma, DWI is the superior imaging technique for the diagnosis of pseudo-responses and that HBDW imaging is particularly advantageous. We also show that RANO- is superior to MacDonald criteria because the size of non-enhanced tumors increases after bevacizumab treatment. On the other hand, as the strong effect of bevacizumab against brain edema may produce a decrease in the T2/FLAIR high intense area, this may hide the extension of the tumor area shown as an increase in the T2/FLAIR high intense area. HBDW imaging clearly demonstrated the extent of the tumor area at an early time point after the start of treatment with bevacizumab.