HER2陽性進行乳癌でtrastuzumab治療を受けた患者の25～34%に脳転移を発症し，治療開始から脳転移の診断までの期間は4～24ヵ月といわれる[28]．trastuzumabの問題は分子量が大きく脳脊髄液への移行は非常にわずかであることから，脳転移や癌性髄膜炎といった中枢神経系転移に対し治療効果を得られない．一方，HER1とHER2の双方を阻害するTKIで，trastuzumabより低分子量のlapatinib(タイケルブ®)は血液脳関門を通過すると考えられており，脳転移に対する効果が期待されている．2006年，進行乳癌に対するlapatinib＋capecitabineとcapecitabine単剤とのRCTで有意に無増悪生存期間(8.4ヵ月vs 4.4ヵ月，P<0.001)が延長したことを報告した中で，発生数が少なく有意差はないものの脳転移の発生が少ない可能性を示唆した[29]．脳転移を有するtrastuzumab治療後のHER2陽性進行乳癌に対する第Ⅱ相試験で，lapatinib単剤による無増悪生存期間は2.7ヵ月，全生存期間は9.6ヵ月であった．また，lapatinib単剤の奏効率は6%で，20%以上の脳転移の縮小は21%に認められ，途中から上記試験の結果を受けて追加されたlapatinib＋capecitabine群で20%以上の脳転移の縮小が40%の症例に認められた[30]．最近報告されたlapatinib plus capecitabine in patients with previously untreated brain metastases from HER2-positive metastatic breast cancer (LANDSCAPE)の結果では，65.9%に50%以上の脳転移の縮小が認められ，無増悪生存期間は5.5ヵ月，評価可能な症例での生存期間中央値は17.0ヵ月と良好な成績を認めた[31]．有害事象としては49%にGrade 3-4の治療関連有害事象を認め，下痢が20%，手足症候群が20%で，31%にいずれかの重篤な有害事象を認めた．治療関連死亡はなかったが9%が毒性中止となった．本試験は脳転移未治療の症例が対象であり，放射線治療を受けるまでの期間は8.3ヵ月であった．

転移性脳腫瘍に対するbevacizumab(アバスチン®)静脈内投与

わが国ではbevacizumabは大腸癌，肺癌，乳癌に対して適応となっているが，当初は転移性脳腫瘍がある症例では重篤な脳出血を認めた症例があったことから禁忌とされていた．その後の報告で脳出血の発症頻度に有意差はないことが報告され，現在は慎重投与に変わっている[32,33]．臨床試験では投与基準が収縮期圧150 mmHg以下と定められており，日常診療でも脳転移を有する症例に対するbevacizumab投与の際には，脳内出血の危険因子である高血圧の管理が重要である．脳転移そのものに対する有効性の報告は乏しいが，SRS後の脳放射線壊死においては有効な報告があることから[34]，宮武らにより2010年4月から，高度医療による多施設臨床試験「症候性脳放射線壊死に対する核医学的診断とベバシズマブの静脈内投与」が行われている[35]．本試験は転移性脳腫瘍を原因疾患とした症候性脳放射線壊死をも対象とした世界初の臨床試験である．現在症例登録を終了し経過観察中で，有効性が証明されれば公知申請により薬事承認の可能性がある．

むすび

脳転移に対する治療もかつてのWBRT中心から，手術，SRT，WBRT，さらには分子標的薬をはじめとする化学療法の組み合わせによる臨床試験のエビデンスが蓄積され治療の選択肢が広がり，ガイドライン上も並列の記載が増えつつある．これらのエビデンスと個々の患者背景，全身状態や神経所見，全身予後などに基づいて腫瘍内科医，放射線治療医，脳神経外科医が協調し最適な治療方針を決定することが重要であると考える．

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RESEARCH Open Access

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.

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.

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).

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

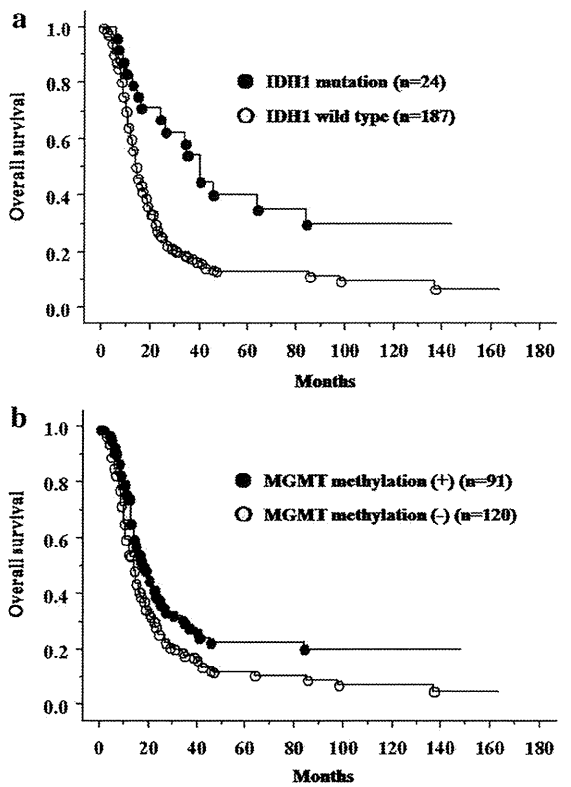

Figure 1 Overall survival for anaplastic astrocytoma and glioblastoma patients. (a) Survival of patients with glioblastoma (GBM) and anaplastic astrocytoma (AA) according to the isocitrate dehydrogenase 1 (*IDH1*) mutation status ($P = 0.0008$). **(b)** Survival of patients with GBM and AA according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

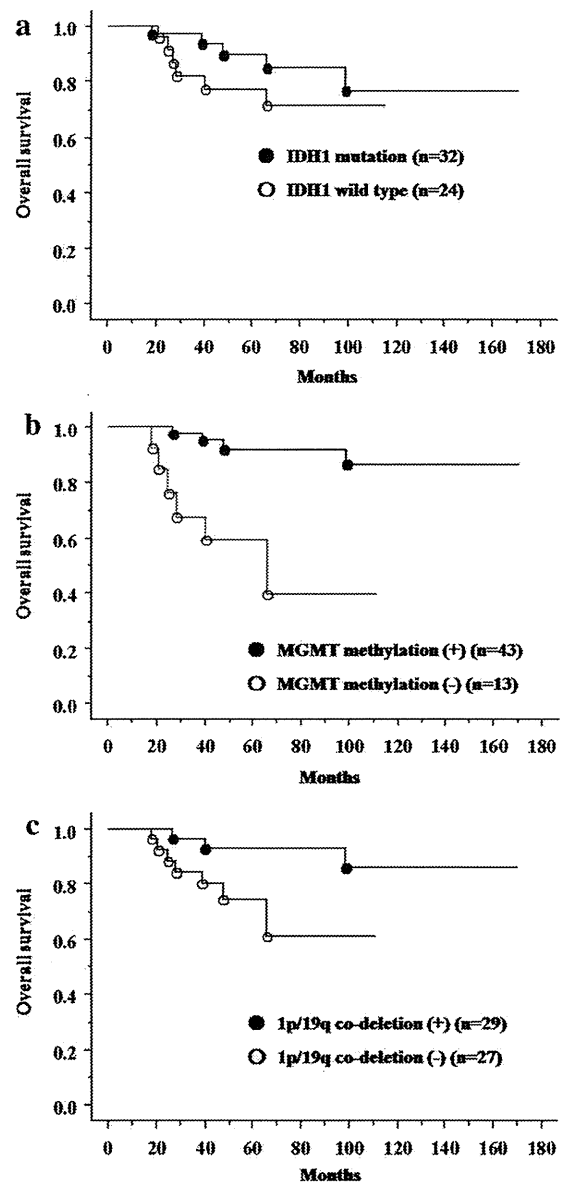

Figure 2 Overall survival for anaplastic oligodendroglioma and anaplastic oligoastrocytoma patients. (a) Survival of patients with anaplastic oligoastrocytoma (AOA) and anaplastic oligodendroglioma (AO) according to the isocitrate dehydrogenase 1 (*IDH1*) mutation (status ($P = 0.3357$). **(b)** Survival 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 ($P = 0.0228$).

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 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

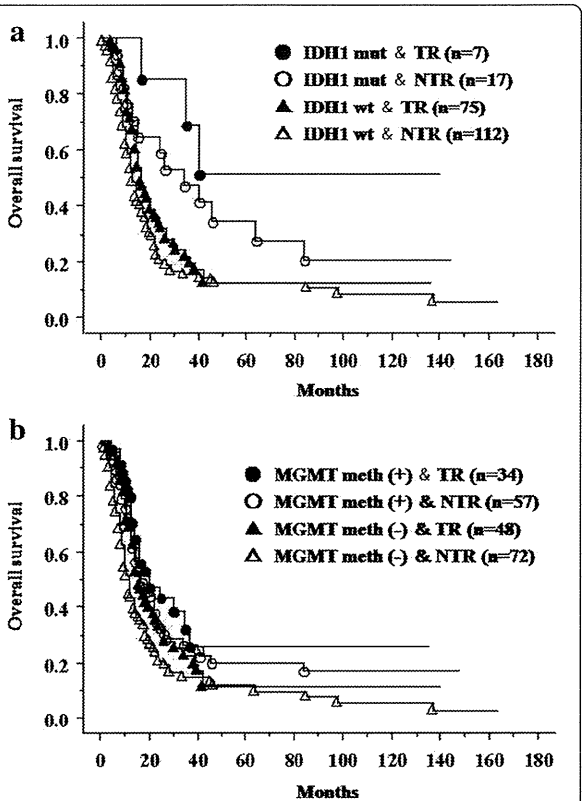

Figure 3 Overall survival for anaplastic astrocytoma and glioblastoma patients according to extent of resection. **(a)** Survival of patients with glioblastoma (GBM) and anaplastic astrocytoma (AA) according to the isocitrate dehydrogenase 1 (*IDH1*) mutation status and extent of resection ($P = 0.0006$). **(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.

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.

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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NEURO-ONCOLOGY

Celecoxib enhances radiosensitivity of hypoxic glioblastoma cells through endoplasmic reticulum stress

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Background. Refractoriness of glioblastoma multiforme (GBM) largely depends on its radioresistance. We investigated the radiosensitizing effects of celecoxib on GBM cell lines under both normoxic and hypoxic conditions.
Methods. Two human GBM cell lines, U87MG and U251MG, and a mouse GBM cell line, GL261, were treated with celecoxib or γ-irradiation either alone or in combination under normoxic and hypoxic conditions. Radiosensitizing effects were analyzed by clonogenic survival assays and cell growth assays and by assessing apoptosis and autophagy. Expression of apoptosis-, autophagy-, and endoplasmic reticulum (ER) stress–related genes was analyzed by immunoblotting.
Results. Celecoxib significantly enhanced the radiosensitivity of GBM cells under both normoxic and hypoxic conditions. In addition, combined treatment with celecoxib and γ-irradiation induced marked autophagy, particularly in hypoxic cells. The mechanism underlying the radiosensitizing effect of celecoxib was determined to be ER stress loading on GBM cells.
Conclusion. Celecoxib enhances the radiosensitivity of GBM cells by a mechanism that is different from cyclooxygenase-2 inhibition. Our results indicate that celecoxib may be a promising radiosensitizing drug for clinical use in patients with GBM.

Keywords: autophagy, celecoxib, ER stress, glioblastoma, hypoxia, radiosensitivity.

Glioblastoma multiforme (GBM) is the most frequently occurring malignant tumor of the central nervous system.[1] Despite standard care comprising maximum surgical resection, 60 Gy of conventional radiotherapy, and chemotherapy with temozolomide (TMZ), the median survival time among patients with GBM is ~1 year.[2]

The molecular mechanisms underlying the initiation and progression of GBM reported to date fall into 2 categories: (i) primary GBM with increased epidermal growth factor receptor (EGFR) expression and mutation of the phosphatase and tensin homolog and (ii) secondary GBM that is transformed from benign glioma types with stepwise mutations in the p53 and retinoblastoma genes. It is known that these genetic alterations in GBM are significantly associated with clinical prognosis.[1] It has also been reported that methylation of O6-methylguanine-DNA methyltransferase increases the susceptibility of GBM tumor cells to alkylating agents, such as TMZ,[3] and mutation in the isocitrate dehydrogenase 1 gene was recently reported to significantly affect the prognosis in patients with GBM.[4]

On the basis of these findings, therapy that targets these specific molecules could be a promising approach for controlling GBM. One possible target is cyclooxygenase-2 (COX-2) because a positive association has been reported among the levels of COX-2, EGFR/EGFRvIII, and activated signal transducer and activator of transcription 3 (STAT3).[5] COX-2 expression was reportedly increased in human GBM and was negatively correlated with clinical outcomes in patients.[6]

Celecoxib, a nonsteroidal anti-inflammatory drug (NSAID), selectively inhibits COX-2.[7] This drug is used clinically both for patients with osteoarthritis and articular rheumatism for pain relief and for patients with familial adenomatous polyposis as an adjuvant to chemotherapy.[8] We previously confirmed that concomitant administration of celecoxib and irinotecan (CPT-11) inhibited the proliferation of neuroblastoma xenografts.[9] However, COX-2 expression in our original neuroblastoma cell lines was low; therefore, there was no clear evidence that celecoxib inhibited COX-2.[9] Thus, we considered the possible existence of

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other pharmacological actions of celecoxib, independent of COX-2 inhibition.[10]

Kardosh et al[11,12] reported that, in addition to its anti-inflammatory and analgesic effects, celecoxib could trigger an endoplasmic reticulum (ER) stress response, as shown by calcium leakage from ER to the cytosol. They also reported that celecoxib exhibited ER stress loading–induced anti-proliferative activity for GBM cell lines.[11] When cells are exposed to endogenous or exogenous stimuli, unfolded proteins accumulate in ER, which engenders unfavorable stress on cells. Although cells respond to reduce ER stress to maintain homeostasis, overwhelming ER stress results in cell death.[13,14] On the basis of these observations, expression of glucose-regulated 78 (GRP78/BiP)[11,15] and expression of C/EBP homologous protein (CHOP/GADD153)[11,16] have been used as markers of the ER stress response.

With regard to clinical trials with celecoxib, Gilbelt et al[17] recently reported that a combination of TMZ and other drugs, including celecoxib, was feasible and safe in a phase I factorial design study. Kesari et al[18] conducted a phase II trial using a combination of TMZ, thalidomide, and celecoxib for patients with GBM. Although they confirmed that these combination protocols were safe, no significant clinical efficacy was found. One reason for this might be that they limited the celecoxib dose to that used for preventing colorectal cancer.[19]

One of the major obstacles to radiotherapy for GBM is the hypoxic conditions of tumor tissues.[20] Hypoxia is also as a known source of ER stress for cells.[21] This suggests that ER stress overloading in hypoxic cells in combination with other ER stress triggers might induce cellular removal through the unfolded protein response pathway.[22] This may be an approach to overcome the radioresistance of tumor cells in hypoxic regions.

Thus, we hypothesized that a combination of 2 ER stress triggers, celecoxib and radiation, might promote GBM cell death even in a hypoxic state. In this study, we examined the radiosensitizing effects of celecoxib on GBM cells in vitro under both normoxic and hypoxic conditions. Establishing the radiosensitizing effects of celecoxib could contribute to developing a novel therapeutic approach for treating patients with GBM.

Materials and Methods

Cell Lines and Normoxic and Hypoxic Cell Culture Conditions

Two human GBM cell lines, U87MG[23] and U251MG,[24] and a mouse GBM cell line, GL261,[25] were obtained from the RIKEN Cell Bank (Tsukuba, Ibaraki, Japan). U87MG cells had wild-type p53,[26,27] whereas U251MG and GL261 cells had mutated p53.[26,28] These cell lines were selected on the basis of COX-2 expression screening by immunoblotting. The p53 and COX-2 expression levels of each cell line are shown in Supplementary Fig. S1. All cells were grown in minimum essential medium (Sigma-Aldrich, Tokyo, Japan) supplemented with 100 mg/mL streptomycin, 100 U/mL penicillin (Sigma-Aldrich), and 10% fetal bovine serum (Sigma-Aldrich). For cell transfer, the cells were rinsed with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS; Sigma-Aldrich) and dispersed with 0.25% trypsin solution containing 0.5 mM ethylenediaminetetraacetic acid (Sigma-Aldrich).

The cells were maintained at 37°C in a humidified incubator. Normoxic conditions were defined as 5% CO_2 in air. Hypoxic conditions were established using a chamber containing AnaeroPack (Mitsubishi Gas Chemical Company, Tokyo, Japan). According to the manufacturer's data sheet, oxygen concentration in the chambers was adjusted to <1% (available at http://www.mgc.co.jp/seihin/a/anaeropack/pdf/pamphlet.pdf [last accessed date 7 April 2013]). The cells were carefully protected from other sources of stress, including nutrient deficiency and confluence. All experiments were concluded before cultures had reached a state of 70%–80% confluence.

Irradiation

Cells were irradiated with ^{137}Cs γ-rays (γ cell 40; Atomic Energy of Canada, Ontario, Canada) at a dose rate of 0.78 Gy/min, which was calculated on the basis of the decay curve of ^{137}Cs. The cells were irradiated under normoxic or hypoxic conditions at room temperature (25–26°C). The doses used were as follows: 2, 4, 6, and 8 Gy for clonogenic survival assays and 6 Gy for cell growth assays, cell cycle analysis, detecting apoptosis and autophagy, and immunoblotting analysis. The dose of 6 Gy was selected on the basis of the results of a clonogenic survival assay, as noted in the Results section.

Chemicals

Celecoxib, 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-pyrazol-1-yl]benzenesulfonamide (Pfizer Co., Ltd., Groton, CT), was dissolved in dimethyl sulfoxide (DMSO; Wako Pure Chemical Industries, Osaka, Japan) at 10 mM and diluted immediately before each experiment. Celecoxib toxicity was assessed using Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kamimasikigun, Kumamoto, Japan) according to the manufacturer's protocol. This method provided for sensitive colorimetric assays to determine cell viability during cell proliferation and cytotoxicity assays (available at http://www.dojindo.com/store/p/456-Cell-Counting-Kit-8.aspx [last accessed date 7 April 2013]). The amount of formazan dye (yellow) generated by the activities of dehydrogenases on a water-soluble tetrazolium salt, WST-8, was directly proportional to the number of viable cells. Each cell line was exposed to celecoxib at 10–70 μM for 48 h under normoxic or hypoxic conditions. For precise assays, DMSO was added to a control sample at the same concentration as that to the celecoxib samples. These experiments were repeated in triplicate. Results were normalized to the value of control cells set at 1 and are presented as means ± standard deviations (SDs).

Clonogenic Survival Assay

A standard colony formation assay was performed immediately after exposing the cell lines to γ-rays. After irradiation, the cells were trypsinized, suspended in PBS on ice, and plated on a 60-mm dish (Falcon, Becton Dickinson, Franklin Lakes, NJ). Colonies were fixed and stained ~14 days later. Three replicate dishes were used for each dose, and colonies of >50 cells were scored as survivors.[29] The doses corresponding to 10% survival (D10) and surviving fractions at 2 Gy (SF2) were determined from a linear quadratic equation (LQ) model that was fit using DeltaGraph5.4 software (RedRock Software Company, Salt Lake, UT).

Doses used were 0, 2, 4, 6, and 8 Gy. Three independent experiments were performed for each dose.

Cell Growth Assay

The effects of celecoxib and/or radiation on tumor cell growth were assessed. Celecoxib concentrations and radiation doses were determined from previous toxicity and clonogenic assays. All cell lines were plated at 1×10^4 cells/well in 24-well plates. When the cells entered the logarithmic growth phase 48 h later, they were treated with celecoxib (50 μM for U87MG and U251MG cells; 30 μM for GL261cells) for 48 h. The cells were then irradiated with γ-rays (6 Gy), after which the medium was immediately changed to fresh control medium. Cell numbers were determined using a Coulter Counter (Beckman Coulter, Tsukuba, Ibaraki, Japan) every 2 days for a period of 10 days after irradiation.

Cell Cycle Analysis

Cells were harvested on days 4, 5, and 10 after celecoxib treatment and used for cell cycle analysis. These time points corresponded to before irradiation (celecoxib alone), 1 day after irradiation, and 5 days after irradiation, respectively. At each time point, the cells were trypsinized and harvested along with the cells floating in the medium. These cells were washed in PBS, fixed in 70% (v/v) ethanol, and stored for up to 2 weeks at −20°C. The cells were then washed once with PBS, followed by incubation in PBS containing 40 μg/mL propidium iodine (Sigma-Aldrich) and 200 μg/mL RNase A (Sigma-Aldrich) for 15 min at room temperature in the dark. Stained nuclei were analyzed using a flow cytometer (BD FACSCalibur; BD Biosciences, San Jose, CA) with 10 000 events/determination. ModFit LT software (Verity Software House Inc., Topsham, ME) was used to assess DNA histograms.

Apoptosis Assay

To detect apoptotic cells, an annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Sigma-Aldrich) was used according to the manufacturer's instructions for flow cytometry analysis. Cells were treated with celecoxib (50 μM for U87MG and U251MG cells; 30 μM for GL261 cells) for 48 h followed by γ-irradiation (6 Gy). Then, the cells were harvested at 6, 48, 72, and 96 h; washed twice in cold PBS; and resuspended in cold binding buffer. Annexin V-FITC (Ann-V) solution (5 μL) and propidium iodide (10 μL) were added to the cell suspension and incubated at room temperature for 10 min in the dark. For each sample, >10 000 cells were examined by flow cytometry.

Autophagy Assay

Autophagy was assessed using a Premo Autophagy Sensors kit (LC3B-FP; Life Technologies, Tokyo, Japan). Cells were placed in a chamber slide under low stress conditions by avoiding nutrient deprivation and confluence. Then, the LC3B reagent was added to the cells and gently mixed according to the manufacturer's instructions. The cells with reagent were incubated overnight (≥16 h) for LC3B expression. Then, cell nuclei were stained with Hoechst 33342 (Dojindo, Kumamoto, Japan) at a final concentration of 8 μg/mL. The cells were examined using a fluorescence microscope (Biozero BZ-8000 KEYENCE; Tokyo, Japan) and incorporated software to obtain Z-stacking images. Because we found that the Premo reagent could not be used to detect autophagosomes in GL261 cells, we assessed GL261 cells with use of a Cyto-ID autophagy detection kit (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer's protocol. Other methods were the same as described above.

For quantitative analysis, acidic vesicular organelles (AVOs) inside cells were visualized and quantified as a marker of autophagy with use of the method described by Kanzawa et al.[30] In brief, after trypsinization, harvested cells were washed once with PBS, resuspended in PBS containing 1 mg/mL acridine orange (Wako Pure Chemical Industries, Osaka, Japan), and incubated for 10 min at room temperature in the dark. The cells were analyzed by flow cytometry; the acid compartment in AVOs was stained red, and the background was stained green with this fluorescent dye. Green (510–530 nm) and red (650 nm) fluorescence emissions from 10 000 cells after excitation with blue light (488 nm) were determined using FACSCalibur (Becton Dickinson).

Immunoblotting

Cells harvested after celecoxib treatment and irradiation were lysed at room temperature in RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, Waltham, MA) with a protease inhibitor cocktail (Halt Protease Inhibitor Cocktail; Thermo Fisher Scientific). The buffer included 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate. Protein concentrations were determined using a 660-nm protein assay kit (Thermo Fisher Scientific) containing Ionic Detergent Compatibility Reagent (Thermo Fisher Scientific). Samples that included the same amounts of protein

were separated by 12.5%–15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by electroblotting on polyvinylidene fluoride membranes (Millipore, Bedford, MA). These blots were incubated with the following antibodies under conditions recommended by the manufacturers. The primary antibodies used were anti-COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-$p21^{Waf1}$ (Santa Cruz Biotechnology), anti-GRP78/BiP (Santa Cruz Biotechnology), anti-GADD153/CHOP (Santa Cruz Biotechnology), anti-microtubule–associated protein 1 light chain 3 (Cosmo Bio, Tokyo, Japan), anti-p53 clone DO-1 (Calbiochem, Darmstadt, Germany), anti-cleaved caspase-3 clone 5A1, and anti-β-actin (Cell Signaling Technology). Horseradish peroxidase–conjugated anti-mouse (Amersham Biosciences, Buckinghamshire, UK) or anti-rabbit antibody (Enzo Life Sciences) was used as the secondary antibody. Signals were detected using an enhanced chemiluminescence system (Millipore, Billerica, MA). Preliminary experiments showed that GRP/BiP, CHOP, and caspase-3 expression was highest at 6 h after each treatment (data not shown); thus, we used data obtained at 6 h in subsequent experiments.

Statistical Analysis

Results are expressed as means ± SDs determined from multiple experiments. Statistical comparisons were made using Student's t tests. $P < .05$ was considered to be statistically significant.

Results

Cell Line Characteristics

p53 function was preserved in U87MG cells, whereas it was absent in U251MG and GL261 cells. COX-2 expression was highest in U87MG cells, followed by GL261 and U251MG cells (Supplementary Fig. S1).

Celecoxib Cytotoxicity and Celecoxib-Induced ER Stress

Celecoxib cytotoxicity results under normoxic and hypoxic conditions as assessed by a CCK-8 assay are shown in Fig. 1A, C, and E. Results for GRP78/BiP expression under normoxic conditions are shown in Fig. 1B, D, and F. These results showed that celecoxib cytotoxicity increased in a dose-dependent manner; however, differences in cell viability between normoxic and hypoxic conditions were insignificant for each cell line investigated. In particular, at celecoxib concentrations of 30 and 50 μM, viability of GL261 cells under normoxic conditions were significantly lower than those of the other cell lines.

Immunoblotting results showed that levels of the ER stress marker GRP78/BiP under normoxic conditions increased as celecoxib concentration increased for each cell line over the concentration range examined (Fig. 1B, D, and F). Cell cytotoxicity and GRP78/BiP expression levels were positively correlated.

On the basis of these results, celecoxib concentration corresponding to a 50% lethal dose for U87MG and U251MG cells was 50 μM, whereas it was 30 μM for GL261 cells under normoxic conditions. Because GRP78/BiP expression significantly increased at either of these concentrations, these concentrations were used in subsequent experiments.

Clonogenic Survival

The results of clonogenic survival assays are shown in Fig. 2. For each cell line, radiosensitivity was significantly reduced under hypoxic, compared with normoxic, conditions. Celecoxib (50 μM for U87MG and U251MG cells; 30 μM for GL261 cells) exhibited significant sensitizing effects under both normoxic and hypoxic conditions, except for U87MG cells.

In addition to evaluations at each dose used, we analyzed the difference between treatment with radiation alone and the combination treatment using SF2 and D10 (Table 1). In this table, SF2 results are presented as means ± SDs and were compared using t tests, whereas D10 results are presented as means ± 95% confidence intervals to determine dose-enhancement ratios. Although the SF2 differences for U87MG cells were insignificant under both normoxic and hypoxic conditions, it was significantly different for the other cell lines under both normoxic and hypoxic conditions. In addition, dose-enhancement ratios calculated at D10 ranged from 1.10 to 1.49 and were higher under normoxic than under hypoxic conditions for each cell line.

Cell Growth

Figure 3 shows the changes in cell number after no treatment, after treatment with celecoxib (50 μM for U87MG and U251MG cells; 30 μM for GL261 cells) or radiation (6 Gy) alone, and after the combination treatment under normoxic and hypoxic conditions. A significant delay in cell growth was observed with the combination treatment under both normoxic and hypoxic conditions. In addition, the combination treatment significantly inhibited cell growth, compared with the treatment with radiation alone under normoxic conditions (Fig. 3B, D, and F). In particular, the effect of combination treatment on GL261 cells was significantly greater under normoxic conditions, and proliferation of these cells was completely arrested after day 12.

Cell Cycle Analysis

DNA histograms of p53 wild-type U87MG cells demonstrated a slight G1 arrest after treatment with radiation alone or the celecoxib plus radiation combination. In contrast, U251MG and GL261 cells, which had mutated p53, did not exhibit G1 arrest; instead, they showed G2/M arrest. In particular, GL261 cells showed strong G2/M arrest after treatment with radiation alone or after the

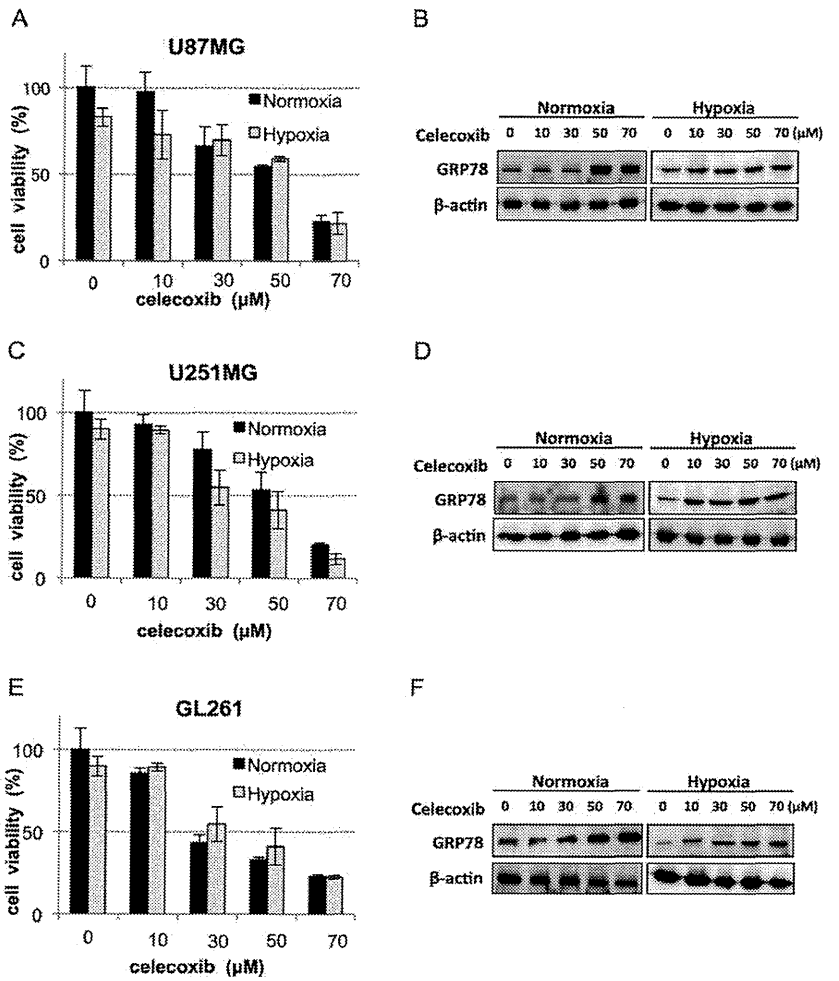

Fig. 1. Cytotoxicity of celecoxib on GBM cells. Cell viabilities of U87MG, U251MG, and GL261 after 48 h of celecoxib treatment are shown by bar graphs (A, C, and E). The black bars indicate normoxic conditions, and the dotted bars indicate hypoxic conditions. The error bars indicate standard deviation calculated from 3 independent experiments. In B, D, and F, expression of GRP78/BiP, a marker of the ER stress, are shown after treatment with celecoxib in concentrations as indicated. Expression of β-actin was used as loading control.

combination treatment (Fig. 4). In addition, G2/M arrest in GL261 cells continued until day 10 after treatment.

Cellular Apoptosis

Figure 5 shows the results for cellular apoptosis after treatment with celecoxib or radiation alone and after treatment with the celecoxib plus radiation combination under normoxic and hypoxic conditions. The proportions of early apoptotic U87MG, U251MG, and GL261 cells under normoxic and hypoxic conditions are shown in Fig. 5A–C. Cellular apoptosis was significantly higher under normoxic than under hypoxic conditions. Treatment with the celecoxib plus radiation combination did not induce any significant increase in apoptosis, compared with treatment with radiation alone, for each cell line.

ER Stress

Figure 5D shows protein expression 6 h after the combination treatment. For each cell line, GRP78/BiP expression levels increased after treatment with celecoxib alone or the celecoxib plus radiation combination. In addition, GRP78/BiP expression levels under hypoxic conditions were higher than those under normoxic conditions, particularly for GL261 cells. GADD153/CHOP expression also increased concomitant with increased GRP78/BiP expression in U87MG and U251MG cells. In addition, GADD153/CHOP expression under hypoxic conditions was significantly increased, compared with that under normoxic conditions, in each cell line. For GL261 cells, GADD153/CHOP expression was increased after combination treatment, compared with treatment with celecoxib alone under hypoxic conditions. Although GADD153/CHOP expression

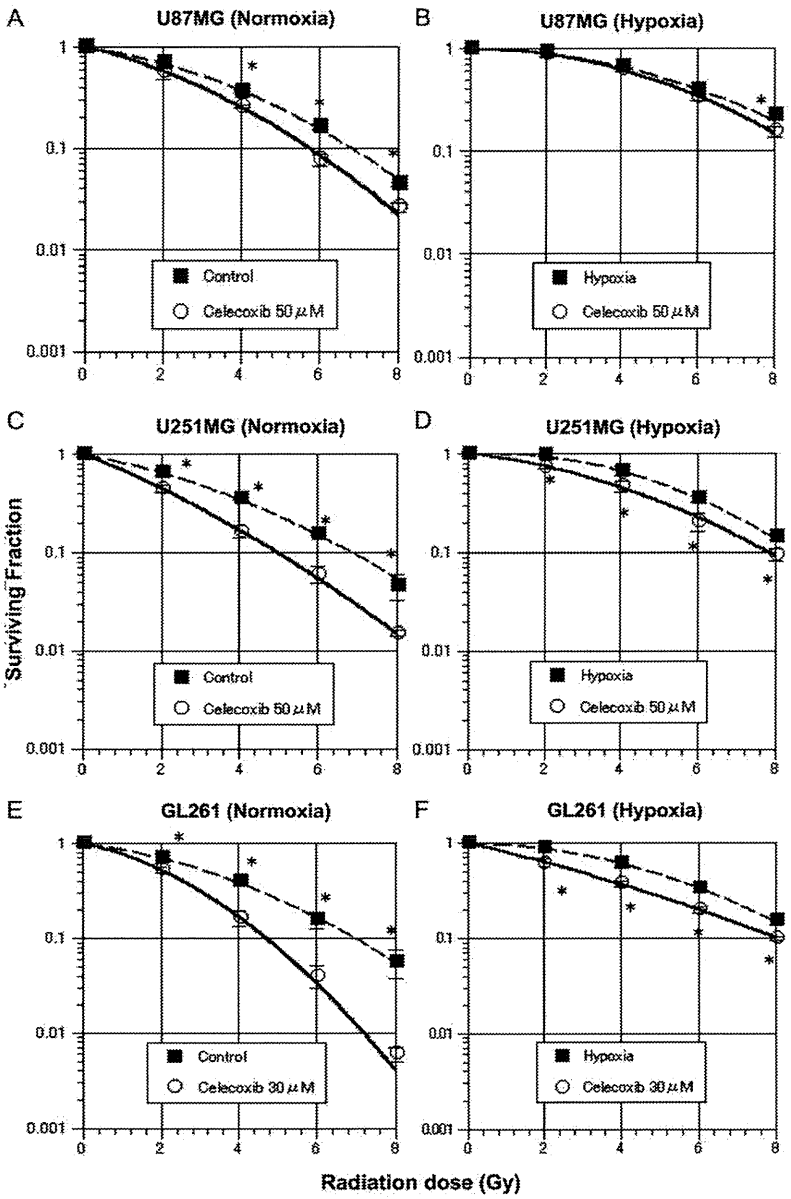

Fig. 2. Clonogenic survival assay. Clonogenic survival curves of U87MG (A: normoxia, B: hypoxia), U251MG (C: normoxia, D: hypoxia), and GL261 (E: normoxia, F: hypoxia) after gamma irradiation with or without celecoxib treatment are shown. The curves were fitted by the linear-quadratic model. Concentrations of celecoxib are 50 μM for U87MG and U251 and 30 μM for GL261. Black squares indicate survival data with celecoxib, and blank squares indicate those without celecoxib. Error bars indicate standard deviations calculated form 3 independent experiments. *$P < .005$ at each dose point.

was enhanced in these cell lines, particularly under hypoxic conditions, cleaved caspase-3 expression was not significantly increased.

Autophagy

Using fluorescence microscopy, we found that the number of autophagosomes in each cell line increased after treatment with celecoxib or radiation alone or after combination treatment. In addition, combination treatment induced a higher number of autophagosomes than treatment with celecoxib or radiation alone. Figure 6A shows representative images of autophagosomes.

The results of flow cytometry analysis to quantify AVOs under normoxic and hypoxic conditions are shown in Fig. 6B–D. In U87MG and U251MG cells, the ratios of AVOs under hypoxic conditions were

Table 1. Surviving fractions at 2 Gy (SF2) and doses for 10% survival (D10) calculated from the survival curves [colcnt=7].

Cell line	SF2			D10		
	Control	Celecoxib	*P*-value	Control	Celecoxib	Dose-enhancement ratio
U87MG						
Normoxia	0.67 ± 0.06	0.60 ± 0.006	$P = .10$	6.88 ± 0.14	5.77 ± 0.06	1.19 ± 0.01
Hypoxia	0.89 ± 0.02	0.88 ± 0.002	$P = .11$	9.70 ± 0.28	8.81 ± 0.07	1.10 ± 0.03
U251MG						
Normoxia	0.66 ± 0.002	0.46 ± 0.03	$P < .01$	6.86 ± 0.04	4.90 ± 0.26	1.40 ± 0.08
Hypoxia	0.96 ± 0.04	0.77 ± 0.04	$P < .01$	8.50 ± 0.17	7.56 ± 0.33	1.13 ± 0.03
GL261						
Normoxia	0.71 ± 0.02	0.51 ± 0.02	$P < .01$	6.94 ± 0.47	4.66 ± 0.21	1.49 ± 0.17
Hypoxia	0.88 ± 0.002	0.63 ± 0.004	$P < .01$	8.80 ± 0.07	8.09 ± 0.07	1.10 ± 0.01

Control: Celecoxib (−).
Celecoxib: 50 μM for U87MG and U251MG, 30 μM for GL261.
Data values: Mean ± SD for SF2, Mean ± 95% CI for D10.

higher than those under normoxic conditions. The number of AVOs was significantly higher with combination treatment than with treatment with celecoxib or radiation alone, and more AVOs were observed under hypoxic than under normoxic conditions for each cell line.

The results for autophagy-related protein LC3 expression are shown in Fig. 6E. A shift from LC3-I to LC3-II was observed with U87MG cells. These results suggest that LC3-II expression was enhanced by celecoxib or radiation alone and combination treatment, compared with the normoxia control. In addition, GRP78/BiP, GADD153/CHOP, and LC3-II expression levels were interrelated in U87MG and U251MG cells after treatment with celecoxib with or without radiation, particularly under hypoxic conditions.

Discussion

GBM is one of the most refractory of tumors and has high radioresistance. Because the effects of ionizing radiation strongly depend on oxygen concentration,[20] radioresistance of GBM may be in part attributable to the presence of hypoxic regions in tumor tissues.[31] This hypothesis is supported, because hypoxia-inducible factor (HIF)−1α is highly expressed in GBM tissues.[32] In addition, a hypoxic environment is known to be a niche for cancer stem-like cells in GBM.[33] Therefore, increasing the radiosensitivity of hypoxic tumor cells is one of the crucial issues to improve the effects of radiotherapy for GBM. In this regard, our results suggest that celecoxib has a radiosensitizing effect not only under normoxic but also under hypoxic conditions.

The original pharmacological action of celecoxib was found to be COX-2 inhibition.[7] COX-2 is a rate-limiting enzyme for cytokines derived from arachidonic acid, and it affects increased production of various cytokines, such as prostaglandin E2, or cancer growth factors, such as TGF-β.[34–36] It has also been reported that COX-2 expression was increased in human GBM and that its expression level was associated with poor clinical prognosis.[37] One reason for this is that prostaglandin E2, a major product of COX-2, accelerates cell motility, growth, invasion, and angiogenesis, whereas it inhibits apoptosis and immune surveillance.[37,38]

Celecoxib reportedly had anti-proliferative activity against colorectal cancer by inhibiting neovascularization.[39] In addition, celecoxib induced apoptosis in colon and prostate cancer cells[40,41] when used at concentrations of ~50–100 μM. Kardosh et al[11] and Du et al[42] reported that this anti-tumor effect was independent of COX-2 inhibition, and Tsutumi et al[43] noted that celecoxib induced tumor cells apoptosis through ER stress overloading.

Stress on the ER, a central cellular organelle with crucial biosynthetic sensing and signaling functions,[44] includes hypoxia,[22] Ca^{2+} depletion,[45] and reactive oxygen species generation.[46] In addition, recent results showed that both radiation and celecoxib could induce ER stress.[11,43,46–48] Although the pharmacological actions of celecoxib that are responsible for inducing ER stress are considered to be involved in suppressing sarcoplasmatic/ER calcium ATPase activity,[48–50] which is independent of a COX-2 inhibitory effect,[51] further analysis is required to establish this point.

We previously reported that a combination of the anticancer drug CPT-11 and celecoxib significantly inhibited the growth of neuroblastomas implanted in mice.[9] We considered that the anti-proliferative activity of this combination treatment was probably caused by inducing strong ER stress[10] because an increase in cellular apoptosis was closely associated with GADD153/CHOP upregulation. The present study results support this hypothesis that was proposed in our previous report.[10]

At present, the signaling pathway for radiation-induced ER stress is considered to be binary, with one pathway involving PERK/eIF2a/CHOP and the other involving IRE1/XBP1/JNK[52] (Supplementary Fig. 2). This binary pathway can result in both cellular apoptosis and autophagy. In addition, ER stress induced by hypoxia and celecoxib is connected to apoptosis or autophagy through this binary pathway.[44,53] Thus, it is conceivable that the combined ER stress induced by radiation,

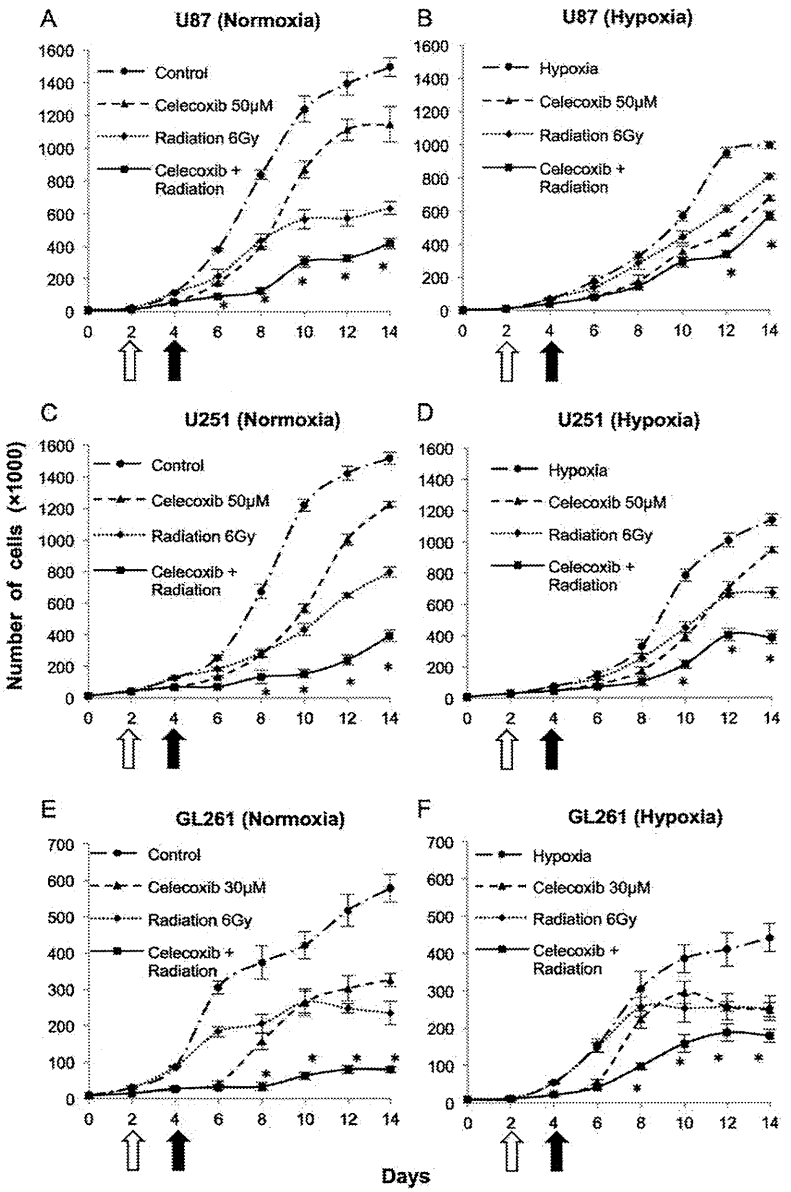

Fig. 3. Cell growth assay. Growth curves of U87MG (A: normoxia, B: hypoxia), U251MG (C: normoxia, D: hypoxia), and GL261 (E: normoxia, F: hypoxia) with radiation or celecoxib treatment alone or with a combination of both are shown. The dose of γ-irradiation is 6 Gy, and concentrations of celecoxib are 50 μM for U87MG and U251 and 30 μM for GL261. The bottom arrows indicate the timings for celecoxib (blank arrows) and radiation treatments (black arrows). Each point represents the mean value, and error bars indicate standard deviation calculated from 3 independent experiments. *$P < .05$ calculated from comparisons between combined treatment and radiation alone.

hypoxia, and celecoxib could be significantly cytotoxic for tumor cells.

With regard to ER stress response markers, GRP78/BiP is a molecular chaperone that is upregulated when ER stress is induced in cells.[15] Moreover, when there is a stress overload, the ER stress response for recovery is overwhelmed and a cell death response is initiated by GADD153/CHOP upregulation.[11,16] In our study, GADD153/CHOP expression in U87MG cells under normoxic conditions was not increased. Although we cannot fully account for this result, the JNK pathway (Supplementary Fig. 2) may be dominant in this cell line under normoxic conditions. Because the other 2 cell lines that we used and U87MG cells under hypoxic conditions exhibited GADD153/CHOP upregulation concomitant with increased autophagy, we consider

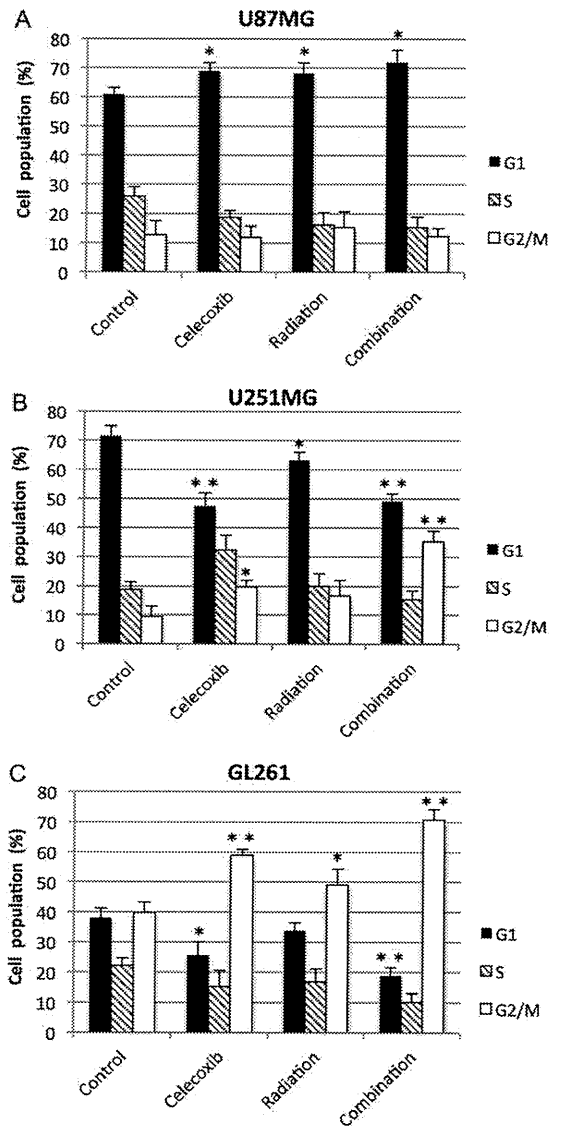

Fig. 4. Cell cycle change of GBM cell lines. The bar graphs show G1 (black bars), S (slash bars), and G2/M (blank bars) populations (%) in DNA histograms analyzed by flow-cytometry in U87MG (A), U251MG (B), and GL261 (C). The data were obtained 24 h after treatments with either celecoxib (50 μM for U87MG and U251 MG and 30 μM for GL261) or radiation (6 Gy) each alone or in combination. Error bars indicate standard deviations calculated from 3 independent experiments. * $P < .05$, ** $P < .01$, calculated from comparisons between control and each treatment sample.

that GADD153/CHOP plays a crucial role in autophagy induction in GBM cells.

In our results, although we observed increased GADD 153/CHOP expression induced by celecoxib/radiation, as noted above, we did not observe caspase-3 upregulation or a significant increase in apoptotic cells (Fig. 5). It is known that ER stress induces not only apoptosis but also autophagy.[54–56] In addition, recent evidence suggests that GADD153/CHOP has a pro-autophagic function and a pro-apoptotic function in the ER stress response,[57] and hypoxia accelerates autophagy by inducting HIF-1 expression.[53] Kang et al[58] recently reported that celecoxib induced autophagy rather than apoptosis, which is compatible with our results.

Clonogenic survival curves indicated that celecoxib significantly enhanced radiosensitivity in each cell line even under hypoxic conditions, except for U87MG cells (Fig. 2). Similarly, the SF2 values were significantly different between treatment with radiation alone and the combination treatment, except for U87MG cells, under both normoxic and hypoxic conditions (Table 1). Furthermore, the growth curves for U87MG cells after treatment with radiation alone and the combination treatment were very close until day 10 (Fig. 3B). These observations indicate that U87MG cells are more resistant to celecoxib than are U251MG and GL261 cells and suggest that the protective ER stress response against celecoxib is greater in U87MG cells than in other cell lines. Although this phenomenon was probably attributable to wild-type p53 preservation in U87MG cells (Supplemental Fig. 1), selective inhibition of p53 in this cell line will be required to establish this hypothesis.

The dose-enhancement ratio at D10 was higher under normoxic than under hypoxic conditions for each cell line (Table 1). Although a CCK-8 assay showed that the cytotoxic effects of celecoxib were not significantly different between normoxic and hypoxic conditions (Fig. 1A, C, and E), the results of clonogenic survival assays suggest that the radiosensitizing effect of celecoxib was less in the hypoxic state than in the normoxic state. Although this difference may be attributable to the strong radiosensitizing effect of oxygen, a dose-enhancement effect of ~10% was observed in each cell line, even in the hypoxic state. We consider that this result has a clinical implication in the treatment of highly refractory GBM.

The results of cell growth assays indicated that celecoxib-induced ER stress also had an inhibitory effect on cell cycle progression. Grosch et al[59] reported that celecoxib modulated cell cycle progression through $P21^{Waf1}$ and $P27^{Kip1}$ upregulation by a mechanism that was independent of COX-2 inhibition. Although we found a significant delay in GBM cell growth after treatment with the radiation plus celecoxib combination, our preliminary results indicated that celecoxib used at the concentrations here did not inhibit COX-2 expression (Supplementary Fig. 3). Therefore, we consider that the proliferative suppression of the GBM cells observed here was most likely caused by ER stress. However, COX-2 inhibition effect might still play a role in tumor cell growth inhibition. This point might be confirmed by clarifying expressions of prostaglandin E2 and/or TGF-β by immunoblotting after exposure to 30 or 50 μM of celecoxib.

In addition, Chen et al[60] reported that prolonged ER stress increased the p27 expression level. This was compatible with our results, because celecoxib alone induced slight G1 arrest in p53 wild-type U87MG cells, whereas it caused G2/M arrest in p53-mutated