別紙４

厚生労働科学研究費補助金（がん臨床研究事業）
分担研究報告書

悪性神経膠腫に対するTemozolomideの治療効果を増強した標準治療確立に関する研究

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研究要旨

希少悪性腫瘍のひとつである悪性神経膠腫の中で、最も予後不良の疾患とされる膠芽腫に対し標準治療となったTemozolomide(TMZ)併用化学放射線療法の治療効果を増強する目的で、Japan Clinical Oncology Group(JCOG)脳腫瘍グループとして、TMZにInterferon-β（INF-β）を併用する化学放射線療法の有効性を評価するランダム化第II相臨床試験を計画し、平成22年4月より24年1月までに目標症例数120（実登録数122）の症例登録を行った。現在2年間の経過観察期間に入っている。また、付随研究として、MGMTと治療効果の関係、10q, 1p, 19q染色体欠失、TP53、CDK2N遺伝子異常、EGFR過剰増幅やPTEN異常などの予後因子を評価するとともに、mRNA, microRNA発現の網羅的解析、SNP-Microarrayによる全染色体の網羅的解析を行うためのプロトコールを作成し、手術検体の回収を行っている。

A～H.の報告内容は研究代表者と同一であるため省略する。

研究成果の刊行に関する一覧

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Cancer Chemother Pharmacol (2013) 71:511–521
DOI 10.1007/s00280-012-2041-5

ORIGINAL ARTICLE

Randomized trial of chemoradiotherapy and adjuvant chemotherapy with nimustine (ACNU) versus nimustine plus procarbazine for newly diagnosed anaplastic astrocytoma and glioblastoma (JCOG0305)

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Received: 6 October 2012 / Accepted: 22 November 2012 / Published online: 11 December 2012

Abstract
Purpose Glioblastoma (GBM) is one of the worst cancers in terms of prognosis. Standard therapy consists of resection with concomitant chemoradiotherapy. Resistance to nimustine hydrochloride (ACNU), an alkylating agent, has been linked to methylguanine DNA methyltransferase (MGMT). Daily administration of procarbazine (PCZ) has been reported to decrease MGMT activity. This study investigated the efficacy of ACNU + PCZ compared to ACNU alone for GBM and anaplastic astrocytoma (AA).

Methods Patients (20–69 years) who had newly diagnosed AA and GBM were randomly assigned to receive radiotherapy with ACNU alone or with ACNU + PCZ. The primary endpoint was overall survival (OS). This was designed as a phase II/III trial with a total sample size of 310 patients and was registered as UMIN-CTR C000000108.
Results After 111 patients from 19 centers in Japan were enrolled, this study was terminated early because temozolomide was newly approved in Japan. The median OS and median progression-free survival (PFS) with ACNU alone ($n = 55$) or ACNU + PCZ ($n = 56$) in the

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intention-to-treat population were 27.4 and 22.4 months ($p = 0.75$), and 8.6 and 6.9 months, respectively. The median OS and median PFS of the GBM subgroup treated with ACNU alone ($n = 40$) or ACNU + PCZ ($n = 41$) were 19.0 and 19.5 months, and 6.2 and 6.3 months, respectively. Grade 3/4 hematologic adverse events occurred in more than 40 % of patients in both arms, and 27 % of patients discontinued treatment because of adverse events.

Conclusions The addition of PCZ to ACNU was not beneficial, in comparison with ACNU alone, for patients with newly diagnosed AA and GBM.

Keywords Glioblastoma · Anaplastic astrocytoma · Nimustine · ACNU · Procarbazine · MGMT

Abbreviations

GBM	Glioblastoma
AA	Anaplastic astrocytoma
ACNU	Nimustine hydrochloride
BCNU	Carmustine
TMZ	Temozolomide
MGMT	Methylguanine DNA methyltransferase
WHO	World Health Organization
PFS	Progression-free survival
OS	Overall survival
RT	Radiotherapy
HR	Hazard ratio
AE	Adverse event
ND	Not determined
CR	Complete response
PR	Partial response
SD	Stable disease
PD	Progressive disease
WBC	White blood cell
3D-CRT	Three-dimensional conformal radiotherapy
CT	Computed tomography
IMRT	Intensity-modulated radiation therapy
BEV	Beam's eye views
DVH	Dose–volume histograms
GTV	Gross tumor volume
CTV	Clinical target volume
PTV	Planning target volume
ICRU	International Commission on Radiation Units
FLAIR	Fluid-attenuated inversion recovery
OAR	Organ-at-risk

Introduction

Glioblastoma (GBM) is one of the worst cancers in terms of prognosis, with almost all patients experiencing progression without cure. According to the report of the Brain Tumor Registry of Japan, the %5-year survival of World Health Organization (WHO) grade IV GBM is 6.9 % and that of WHO grade III anaplastic astrocytoma (AA) is 33.9 % [1].

Standard therapy against GBM consists of the maximal resection that is safely possible, with concomitant chemo-radiotherapy. Currently, temozolomide (TMZ) is the

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standard agent used in the treatment of GBM. However, before the TMZ era, nitrosourea had been widely used for GBM and AA. The Glioma Meta-analysis Trialists Group described that chemotherapy including nitrosourea showed significant prolongation of survival, with a hazard ratio of 0.85 ($p < 0.0001$) [2].

Nimustine hydrochloride (ACNU) was developed in Japan, and for more than 20 years since 1980, it has been the standard chemotherapeutic agent against gliomas [3]. Wolff et al. [4] analyzed 364 studies, including a total of 24,193 patients with high-grade glioma, and reported that the survival gain in the 15 ACNU-treated cohorts was 8.9 months, compared to those who received different drugs or no chemotherapy. Takakura et al. [5] reported that the overall survival (OS) of AA and GBM treated by radiotherapy (RT) and concomitant ACNU were 36 and 12 months, respectively. Furthermore, the response rate of a more than 50 % reduction in tumor size was 46.2 % in both AA and GBM. Alkylating agents, including ACNU and procarbazine (PCZ), confer cytotoxic effects on glioma cells by alkylation at the O^6-position of guanine in DNA. This results in the formation of DNA cross-links [6]. Methylguanine DNA methyltransferase (MGMT) removes methylation damage induced by nitrosourea from the O^6-position of DNA guanines before cell injury, and this enzyme was detectable in 76 % of glioma tissues [7]. MGMT in glioma cells is a primary defense against nitrosourea, but the cellular methyltransferase activity of MGMT is exhausted after MGMT takes effect. Daily administration of PCZ for 10 days was reported to cause the accumulation of O^6-methylguanine; it also decreased MGMT activity in rat liver [8] and lymphocytes in lymphoma patients [9]. Inhibition of MGMT by O^6-benzylguanine increased the cytotoxicity of TMZ and carmustine (BCNU) to tumor cells [10]. From these results, it can be predicted that daily administration of PCZ, by depleting MGMT activity, will increase the efficacy of ACNU against AA and GBM.

To prove this hypothesis and establish a more potent standard therapy for AA and GBM, the Brain Tumor Study Group of the Japan Clinical Oncology Group (JCOG) conducted this clinical trial. The study was terminated at the end of the phase II part. The current report describes the final outcome of the study.

Subjects and methods

Patient eligibility criteria

Patients aged 20 to less than 70 years of age who had newly diagnosed and histologically proven supratentorial GBM or AA were eligible for this study. Patients were enrolled between 3 and 14 days after their operation. To be eligible, a patient's preoperative MRI had to show that more than 50 % of the tumor was located in supratentorial areas, except the optic nerve, olfactory nerve, or pituitary gland. Eligible patients had Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–2 or 3 (only in cases with neurologic symptoms caused by a tumor) and adequate hematologic, pulmonary, renal, and hepatic function, defined as follows: white blood cell (WBC) count $\geq 3.0 \times 10^3$/mL, hemoglobin level ≥ 8.0 g/dL, platelets count $\geq 1.0 \times 10^6$/mL, aspartate transaminase (AST) level ≤ 100 IU/L, alanine transaminase (ALT) level ≤ 100 IU/L, serum creatinine level ≤ 1.0 mg/dL. Additionally, written informed consent was obtained from all the participating patients. We excluded patients with multiple or disseminated tumors or large tumors in which the planned target volume for irradiation exceeded 1/3 of the whole-brain volume. Additionally, we also classified as ineligible any patient who was pregnant, had meningitis, pneumonia, diabetes mellitus with insulin injection, myocardial infarction, or unstable angina pectoris within the last 3 months, mental disorders, a history of pulmonary fibrosis or interstitial pneumonia, or other forms of active cancer occurring within 5 years of treatment. The study protocol was approved by JCOG Protocol Review Committee and institutional review board at each center.

Treatment

After the confirmation of the eligibility criteria, registration was made by telephone or fax to the JCOG Data Center. Patients were randomized within 14 days of surgery to either ACNU with RT (the control arm, A) or to ACNU + PCZ with RT (the experimental arm, B) (Fig. 1a) by a minimization method with adjustment factors consisting of histology (GBM vs. AA), age (younger than 60 vs. 60 years or older), residual tumor (presence vs. absence), and institution. Residual tumor was assessed using a gadolinium-enhanced MRI obtained within 72 h of the surgery.

Radiotherapy with concomitant chemotherapy was started within 3 weeks after the surgery. Patient positioning and immobilization with an individual head mask and computed tomography (CT)-based planning were required. Treatment was delivered using linear accelerators with nominal energies ≥ 4 MV. Intensity-modulated radiation therapy (IMRT) technique was not permitted. All fields were to be treated every day. Three-dimensional conformal radiotherapy (3D-CRT) planning including the use of beam's eye views (BEV) and dose–volume histograms (DVH) were recommended for volumetric dose evaluation. Quality assurance reviews were done at the Radiotherapy Support Centre in Tokyo, Japan, with feedback sent to each

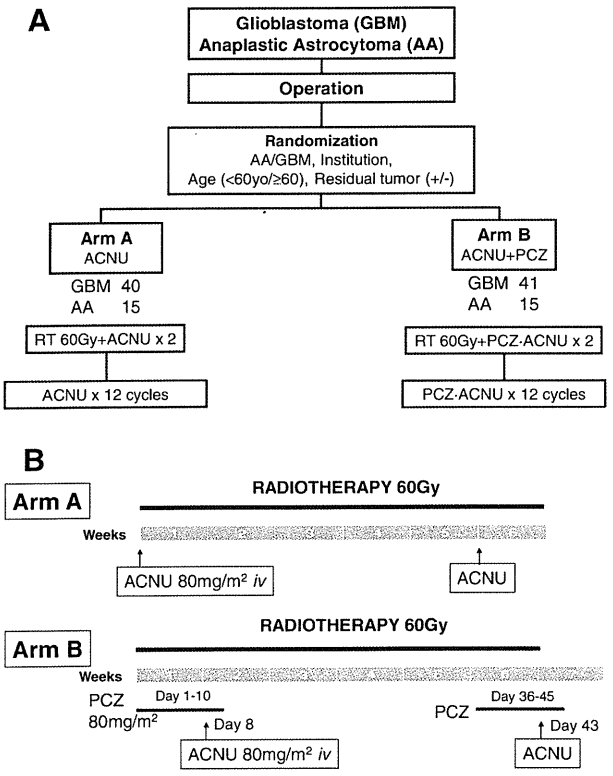

Fig. 1 **a** Study design of JCOG 0305: RT + ACNU versus RT + ACNU + PCZ; 40 patients with GBM and 15 patients with AA were assigned to *arm A*, and 41 patients with GBM and 15 patients with AA were assigned to *arm B*. **b** Treatment schedule of RT + ACNU (*Arm A*) and RT + ACNU + PCZ (*Arm B*)

institution by the radiotherapy study coordinator (Minako Sumi). The minimum and maximum dose to the PTV should be comprised between 95 and 107 % of the International Commission on Radiation Units (ICRU) reference point dose. The gross tumor volume (GTV) was defined as the primary tumor with or without enhancement on CT or magnetic resonance imaging (MRI). The clinical target volume 1(CTV1) included GTV, the resection cavity and surrounding edema (high-intensity area on T2-weighted or fluid-attenuated inversion recovery (FLAIR) image) plus a 1.5-cm margin. The CTV2 included GTV and the resection cavity plus a 1.5-cm margin. Planning target volume (PTV) was defined as CTV plus a margin of 0.5 cm or more. The doses for PTV1 and PTV2 were 50 and 10 Gy, respectively. The protocol required contouring organ-at-risk (OAR), including optic chiasm, brain stem, and retina. Cumulative doses to the optic chiasm and brainstem were limited to a maximum dose of 50 and 45 Gy for the retina.

In the control arm A, 80 mg/m^2 of ACNU was administered intravenously on days 1 and 36 during RT (Fig. 1b). In the experimental arm B, 80 mg/m^2 of oral PCZ was administered daily from days 1 to 10 and days 36 to 45, and given together with intravenous ACNU (80 mg/m^2) on days 8 and 43. Adjuvant therapy consisting of 80 mg/m^2 of ACNU alone in arm A or ACNU plus PCZ (PCZ: 80 mg/m^2 orally on days 1–10, ACNU: 80 mg/m^2 intravenously on day 8) in arm B started 56 days from the final administration of ACNU and was given every 8 weeks, for up to 12 cycles. Doses of ACNU and PCZ were calculated using actual body surface area, reduced for toxicity, and were not escalated.

Evaluations and follow-up

Baseline and follow-up examinations included vital signs, subjective symptoms, neurologic examination, MRI scan, and blood and serum laboratory examinations. For each patient, these examinations were performed weekly, with the exception of MRI scans, which were performed between the end of the initial chemoradiotherapy and the beginning of adjuvant therapy. All examinations were performed before each cycle of adjuvant chemotherapy, at a frequency of nearly every 2 months. After completion of the treatment protocol, patients were assessed every 3 months until progression. Toxicity was graded using the National Cancer Institute Common Toxicity Criteria (version 2). Findings of radiation necrosis were also assessed on MRI. Each patient was required to undergo a follow-up examination for at least 2 years from the date of randomization.

Tumor progression on MRI was defined according to Response Evaluation Criteria in Solid Tumors (RECIST), version 1.0 [11]. Progression of disease was defined as a 20 % increase in tumor size, as shown by contrast-enhanced imaging, or the development of new lesions, neurologic deterioration, or death by any cause. Further treatment at recurrence or progression was discretionary, but recorded.

A central pathology review by 3 independent pathologists (Yoichi Nakazato, a member of the Working Group for WHO 2007 classification; Nobuaki Funata; and Toru Iwaki) was performed and determinations given. A central review of radiological response was also performed.

Statistical analysis

When we planned this study, TMZ had been widely approved and was used worldwide. However, TMZ was not available in Japan. ACNU remained the standard therapy in Japan, but there was no sufficient data regarding this treatment. We planned a phase II/III clinical trial, with the phase II part designed to confirm the feasibility of ACNU and ACNU + PCZ.

The primary and secondary endpoints for the phase II part were %6-month survival and adverse events (AEs) in ACNU + PCZ arm. The primary endpoint of the phase III part was OS, while the secondary endpoints were PFS, response rate, complete response rate, and AEs.

Overall survival was calculated from the date of random assignment to the date of death from any cause and censored at the last follow-up for event-free patients. PFS was calculated from the date of randomization to the date of progression or death from any cause and censored at the last verifiable progression-free date for event-free patients. OS and PFS were estimated by the Kaplan–Meier method. OS was analyzed by the stratified log-rank test for eligible patients with adjustment factors, excluding institution. Unstratified log-rank tests were used for the analysis of PFS and subgroup analyses of OS and PFS. Fisher's exact test was used for categorical data. All p values are two-sided, except for primary analysis of OS.

We assumed %2-year survivals in AA and GBM for arm A were 50 and 20 %, and the ratio of those patients with AA or GBM enrolled in this study was expected to be 2:3. The phase III study was designed to enroll 155 patients per arm with 5 years of accrual and 2 years of follow-up, including those for the phase II part and about 10 % of ineligible patients, to achieve at least 75 % power to detect a hazard ratio (HR) of 0.74, with a one-sided alpha of 0.05 [12].

Three interim analyses were planned. The first was planned during phase II to test whether %6-month survival in arm A was superior to the predefined threshold (80 %), with a one-sided alpha of 0.1 and beta of 0.2, when 56 patients were included in ACNU + PCZ arm. The second and third interim analyses of OS were planned during phase III. For analyses of phase III part, multiplicity was adjusted by the Lan and DeMets alpha-spending function with the O'Brien and Fleming stopping boundary to control the type I error for primary endpoint.

In March 2007, protocol was amended to stop patient accrual after 111 patients had enrolled and to carry out the final analysis without planned interim analyses for both of phase II and phase III part. This was done because toxicity of both arms was unexpectedly high in phase II and because TMZ became available in Japan.

All statistical analyses were performed using SAS software, release 9.1 (SAS Institute, Cary, NC).

This trial is registered with UMIN-CTR (www.umin.ac.jp/ctr/), number C000000108.

Results

Patient characteristics

A total of 111 patients from 19 centers were randomly assigned to arm A ($n = 55$) or arm B ($n = 56$) from March 2004 to September 2006. Primary analyses were performed in September 2007, and the updated analyses were completed in July 2009. All patients were eligible for this study. Baseline characteristics were well balanced between the arms (Table 1). The median ages for arms A and B were 56 and 54 years, respectively. Total numbers for AA and GBM patients were 15 (27.3 %) and 40 (72.7 %) in arm A and 15 (26.8 %) and 41 (73.2 %) in arm B. PS 0 to 1 in arms A and B were 45 (81.8 %) and 41 (73.2 %), respectively. Eighteen (32.7 %) patients in arm A and 21 (37.5 %) patients in arm B underwent gross total removal, and no residual tumor was confirmed on MRI scans. The median duration of follow-up was 20.2 (range 0–48.0 months) for all eligible patients.

Treatment

Patient compliance with the treatment regimen is depicted in Table 2. Among the 111 total patients, 1 patient in arm A died from pulmonary embolism before the beginning of initial chemoradiotherapy. Fifty-three (96.3 %) patients in arm A completed initial chemoradiotherapy and received ACNU twice. In arm B, 48 out of 56 (85.7 %) patients received 2 cycles of PCZ + ACNU, and 8 patients (14.3 %) received 1 cycle of PCZ + ACNU in initial chemoradiotherapy. Eighteen (32.7 %) patients in arm A and 20 (35.7 %) patients in arm B failed to start adjuvant chemotherapy. Furthermore, 14 (25.5 %) patients in arm A and 23 (41.1 %) patients in arm B discontinued protocol therapy by the fourth cycle of adjuvant chemotherapy. The numbers of patients who received 4 cycles of chemotherapy or more were 23 (41.8 %) and 13 (23.2 %) in arm A and B, respectively. Only 5 (9.1 %) patients in arm A and 2 (3.6 %) patients in arm B completed the full protocol therapy. Nineteen (34.5 %) patients in arm A and 22 (39.3 %) patients in arm B discontinued the protocol for reasons other than completion of protocol or disease progression [arm A: 31 (56.4 %), arm B: 32 (57.1 %)]. Reasons for discontinuation were as follows: AEs [arm A: 6 (10.9 %), arm B: 13 (23.2 %)]; patient refusal related to AE

Table 1 Baseline characteristics in the ITT population

	Arm A ($n = 55$) (RT + ACNU)	Arm B ($n = 56$) (RT + PCZ + ACNU)
Age	56 (24–69)	54 (24–69)
Sex		
Male	32 (58.2 %)	33 (58.9 %)
Female	23 (41.8 %)	23 (41.1 %)
PS		
0, 1	45 (81.8 %)	41 (73.2 %)
2, 3	10 (18.2 %)	15 (26.8 %)
Histology		
Grade 3 (AA)	15 (27.3 %)	15 (26.8 %)
Grade 4 (GBM)	40 (72.7 %)	41 (73.2 %)
Surgery		
Gross total removal	18 (32.7 %)	21 (37.5 %)
Partial removal	30 (54.5 %)	26 (46.4 %)
Biopsy only	7 (12.7′ %)	9 (16.1 %)

Table 2 Compliance

	Arm A ($n = 55$) (RT + ACNU)	Arm B ($n = 56$) (RT + PCZ + ACNU)
RT (completion)	54 (98.2 %)	56 (100 %)
Initial chemotherapy		
1 cycle	1 (1.8 %)	8 (14.3 %)
2 cycles	53 (96.4 %)	48 (85.7 %)
Adjuvant chemotherapy		
None	18 (32.7 %)	20 (35.7 %)
1–3 cycles	14 (25.5 %)	23 (41.1 %)
4–6 cycles	9 (16.4 %)	7 (12.5 %)
7–11 cycles	9 (16.4 %)	4 (7.1 %)
12 cycles (completion)	5 (9.1 %)	2 (3.6 %)

[arm A: 3 (5.5 %), arm B: 8 (14.3 %)]; and patient refusal not related to AE [arm A: 7 (12.7 %), arm B: 1 (1.8 %)].

After discontinuation of the protocol, 28 (50.9 %) patients in arm A and 29 (51.8 %) patients in arm B received TMZ as further treatment.

Central review of histology

The central pathology review diagnosis of all cases was performed according to the WHO 2007 classification (Table 3). Among 81 GBM in the intention-to-treat (ITT) population, 69 (85.2 %), 4 (4.9 %), 3 (3.7 %), 2 (2.5 %), and 3 (3.7 %) were diagnosed as GBM, anaplastic oligoastrocytoma (AOA), anaplastic oligodendroglioma (AO), AA, and others, respectively. Only 10 cases (33.3 %) were diagnosed as AA among the 30 AA in the ITT population, while 8 (26.7 %), 5 (16.7 %), 3 (10.0 %), 2 (6.7 %), and 2 (6.7 %) were confirmed as GBM, AOA, diffuse astrocytoma, pilocytic astrocytoma, and others, respectively. Among all 111 cases, phenotype change of astrocytic to oligodendroglial tumor occurred in 14 cases (12.6 %). Finally, 77 and 12 patients were diagnosed with GBM and AA, respectively, by central pathology review.

Response rate

The overall radiographic response rate for 66 measurable diseases after surgery, based on RECIST guideline, was assessed by Diagnostic Radiology Committee. The response rate was 21.2 % (7/33) in arm A and 6.1 % (2/33) in arm B. In GBM patients from the ITT population, response rates were 25.0 % in arm A [3 CR (complete response), 2 PR (partial response), 1 SD (stable disease), 14 PD (progressive disease)] and 9.1 % in arm B [1 CR, 1 PR, 1 SD, 19 PD] ($p = 0.23$). In AA patients from the ITT population, response rates were 15.4 % in arm A (2 PR, 1 SD, 10 PD) and 0 % in arm B (11 PD) ($p = 0.48$).

Progression-free survival

In the entire ITT population, PFS was 8.6 months [95 % confidence interval (CI); 5.1–20.5] in arm A ($n = 55$, 44 events), compared with 6.9 months (95 % CI 5.1–9.0, $p = 0.36$) in arm B ($n = 56$, 47 events) (Fig. 2a). According to grades in the ITT population, PFS of GBM in arm A ($n = 40$, 33 events) and B ($n = 41$, 35 events) was 6.2 (95 % CI 4.2–13.2) and 6.3 months (95 % CI 3.0–8.9), respectively ($p = 0.35$) (Fig. 2b). PFS of AA in arm A ($n = 15$, 11 PD) and B ($n = 15$, 12 events) was 8.6 (95 % CI 5.1–35.4) and 9.0 months (95 % CI 5.8–21.8), respectively ($p = 0.83$) (Fig. 2c). No difference was observed between the arms in any subgroup defined by histology, presence of remaining tumor, or age under or over 60.

In the subgroup defined by central pathology review, the PFS of GBM in arms A ($n = 37$, 33 events) and B ($n = 40$, 36 events) was 5.1 (95 % CI 4.2–10.3) and 5.7 months (95 % CI 2.7–8.4), respectively ($p = 0.49$) (Fig. 2d). The PFS of AA and AOA in arm A ($n = 9$, 4 events) and B ($n = 12$, 9 events) was ND (not determined) and 7.9 months (95 % CI 5.2–22.5), respectively ($p = 0.21$).

Table 3 Local diagnosis and central pathology review

Grade	Histology	Local diagnosis	Central pathology review		
			Total	Arm A	Arm B
IV	*Glioblastoma*	83	77	37	40
III	*Anaplastic astrocytoma*	30	12	6	6
III	Anaplastic oligoastrocytoma		9	3	6
III	Anaplastic oligodendroglioma		3	2	1
III	Anaplastic ependymoma		1	1	0
II	Diffuse astrocytoma		4	1	3
II	Oligoastrocytoma		1	1	0
II	Oligodendroglioma		1	1	0
I	Pilocytic astrocytoma		2	2	0
	Sarcoma		1	1	0
	Total	111	111	55	56

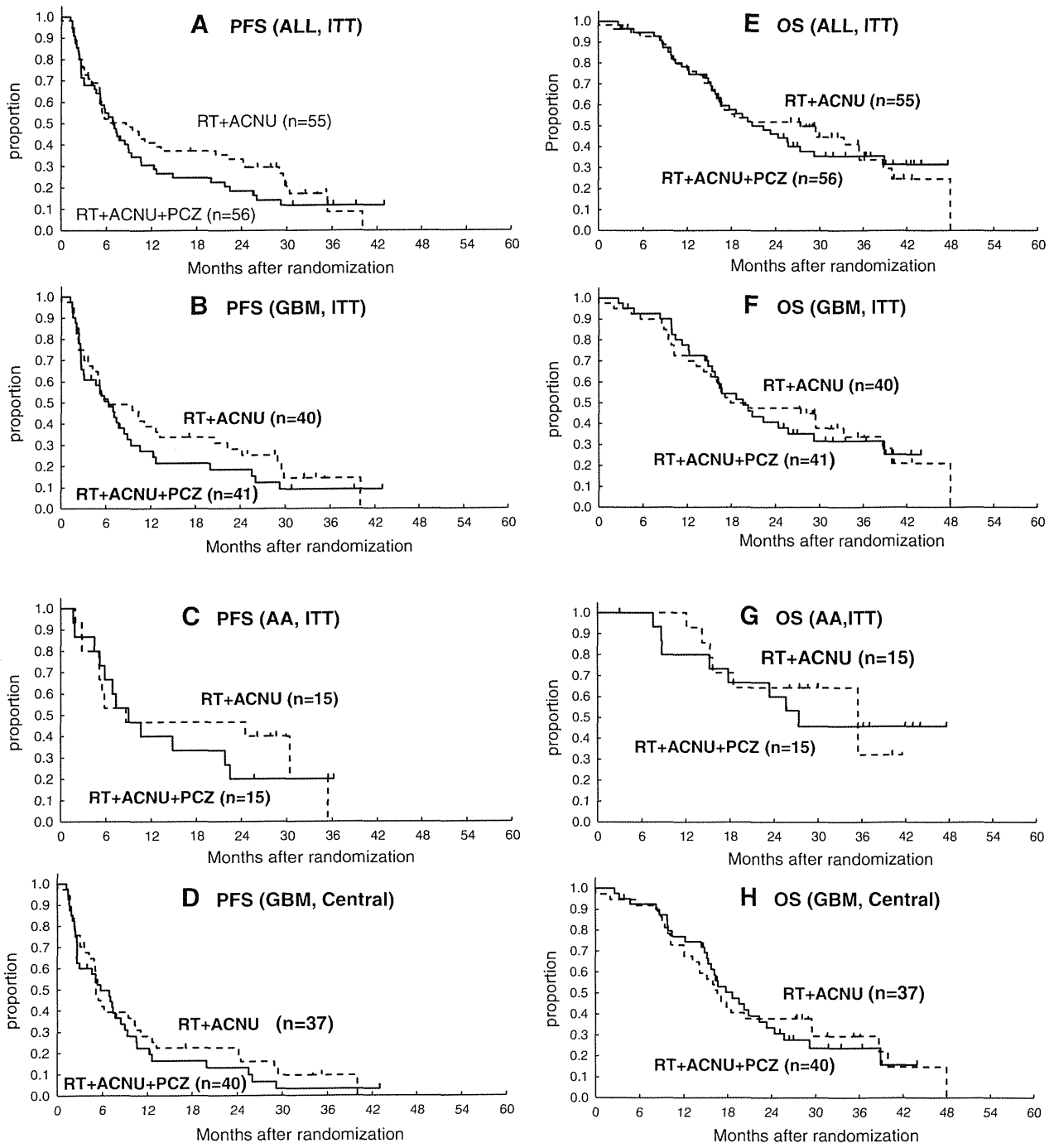

Fig. 2 Progression-free survival in the intention-to-treat (ITT) population (**a**), the GBM subgroup (**b**), the AA subgroup (**c**), and the GBM subgroup with central pathology review (**d**). Overall survival in the ITT population (**e**), the GBM subgroup (**f**), the AA subgroup (**g**), and the GBM subgroup with central pathology review (**h**). RT + ACNU alone (*solid line*), RT + ACNU + PCZ (*dotted line*)

Overall survival and cause of death

From the entire ITT population, 35 patients died in each group. In arm A ($n = 55$), OS was 27.4 months (95 % CI 16.2–35.4), compared with 22.4 months (95 % CI 16.4–29.2) in arm B ($n = 56$) (Fig. 2e). The %2-year survival in arms A and B was 51.9 % and 46.2 %, respectively. There was no difference between the 2 arms ($p = 0.75$ and pre-planned, one-sided $p = 0.62$, by stratified log-rank test).

The OS of GBM subgroup in arms A ($n = 40$, 28 death) and B ($n = 41$, 27 death) was 19.0 (95 % CI 15.2–33.3) and 19.5 months (95 % CI 15.8–29.2), respectively ($p = 0.90$) (Fig. 2f). The %2-year survival in arms A and B was 48 and 41 %, respectively. The OS of AA subgroup in arms A ($n = 15$, 7 death) and in arm B ($n = 15$, 8 death) was 35.4 [95 % CI 15.7–not estimated (NE)] and 27.4 months (95 % CI 17.8–NE), respectively ($p = 0.88$) (Fig. 2g). There were no differences between the arms of any subgroup.

In the subgroups defined by central pathology review, the OS of GBM in arm A ($n = 37$, 28 death) and in arm B ($n = 40$, 29 death) was 16.6 (95 % CI 13.3–29.5) and 18.7 months (95 % CI 15.4–23.4), respectively ($p = 0.92$) (Fig. 2h). The %2-year survival in arms A and B was 38 and 34 %, respectively. The OS of AA and AOA in arm A ($n = 9$, 3 death) and B ($n = 12$, 4 death) was 33.3 months (95 % CI 15.7–33.3) and NE, respectively ($p = 0.83$).

Among the 70 total deaths, 31/35 (88.6 %) patients in arm A and 32/35 (91.4 %) in arm B experienced neuronal death of an original tumor. One patient (2.9 %) in arm A and 2 (5.7 %) patients in arm B contracted treatment-related pneumonia and died from that illness. Other causes of death were pulmonary embolism (1), pneumonia (2), and unknown (1).

Toxicity

Toxicity was assessed in 110 patients receiving initial therapy and in 73 patients receiving adjuvant chemotherapy. The most frequent grade 3/4 toxicities, experienced by more than 10 % of patients, were hematologic, neurologic, gastrointestinal, and hepatic AEs (Table 4). Patients in both arms frequently experienced leukopenia and neutropenia; more than half of the patients in arm B experienced these AEs during adjuvant therapy as well as during initial therapy. More than 40 % of patients in arm A also experienced these hematologic events even during adjuvant therapy. Grade 4 neutropenia was observed in 5.6 and 39.3 % of patients in arms A and B during initial chemoradiotherapy and 11.1 and 15.6 % during adjuvant therapy. Grade 3/4 nausea and anorexia were seen in 10.7 and 16.1 % of patients in initial therapy in arm B, but were rare in the adjuvant-therapy subgroups in both arms. One patient in arm B had cerebral infarction. Extrapyramidal signs, including tremors or involuntary movements, occurred in 2 patients in each arm.

Grade 3/4 pneumonitis occurred in 1 patient in arm A and 2 in arm B during the entire treatment period. Opportunistic infections—including 2 cases of *Pneumocystis jirovecii* pneumonia (PCP), 1 case of oral candidiasis, and 2 case of herpes zoster—occurred in arm B. One patient (1.8 %) in arm A and 2 (3.6 %) patients in arm B died from treatment-related pneumonia, and 1 of these patients in arm B had PCP. One patient in arm B died from sepsis and acute respiratory distress syndrome after initial therapy. One patient in arm A died from pulmonary embolism before starting chemoradiotherapy, and 1 patient in arm A and 2 patients in arm B died from pneumonia following tumor progression.

Radiation necrosis was observed in 2 out of 54 (3.7 %) patients in arm A and 1 out of 56 (1.8 %) patients in arm B. During surgery, 1 patient in arm A was found to have radiation necrosis. Pseudo-progression within 3 months after chemoradiotherapy was not suspected in any patient.

Discussion

This study aimed to evaluate the efficacy and safety of treatment with ACNU + PCZ compared to ACNU alone as concomitant chemoradiotherapy against AA and GBM. We found no obvious differences in OS or PFS for AA and GBM between the treatment groups, but patients treated with ACNU + PCZ experienced more adverse effects than those treated with ACNU alone. TMZ is an effective regimen for malignant gliomas with less toxicity than our ACNU regimens, but it was not approved in Japan when this study began. At the end of the phase II part of this study, TMZ became available even in Japan, so this study was terminated at that point.

Methylguanine DNA methyltransferase is a major DNA repair protein and is implicated in resistance of glioma cells to alkylating agents [13]. Transcriptional silencing by MGMT promoter methylation results in inhibition of MGMT expression [14], and thus MGMT promoter methylation is strongly associated with survival in glioma patients treated with either nitrosourea or TMZ [15–17]. The status of the promoter of MGMT in primary tumors was frequently observed to change from methylated to unmethylated in recurrent tumors following ACNU or TMZ treatment [18, 19], which constitutes one of the mechanisms behind malignant gliomas' resistance to nitrosourea and TMZ. The rationale for treatment with ACNU + PCZ is that daily application of PCZ depletes MGMT activity, increasing sensitivity of AA and GBM to ACNU. Dose-dense TMZ therapy based on the theory of depletion of MGMT [20, 21], or BCNU or TMZ with direct inhibition of MGMT by O^6-benzylguanine [22, 23] has been shown in previous studies to be effective for GBMs. However, there was no difference in OS found between standard and dose-dense TMZ for newly diagnosed GBMs [20].

While we were conducting this study, the European Organization for Research and Treatment of Cancer (EORTC) Brain Tumor and Radiotherapy Groups and the

Table 4 Toxicity

Grade 3/4 adverse events	Initial therapy with RT ($n = 110$) (%)		Adjuvant therapy ($n = 73$) (%)	
	Arm A	Arm B	Arm A	Arm B
Hematologic				
Leukopenia	38.9	73.2	40.5	69.4
Neutropenia	38.9	76.8	44.4	56.3
Thrombocytopenia	5.6	50.0	40.5	50.0
Anemia	0	8.9	10.8	8.3
Neurologic				
Seizure	9.3	7.1	5.4	8.8
Speech impairment	11.1	10.7	5.4	2.9
Neuropathy-motor	11.1	12.5	0	0
Extrapyramidal sign	0	0	5.4	2.7
Pulmonary (pneumonitis)	0	3.6	2.7	0
Gastrointestinal				
Nausea	0	10.7	0	0
Anorexia	1.9	16.1	0	2.9
Hepatic				
AST	3.7	12.5	2.9	2.9
ALT	3.7	16.1	2.9	8.8
Total bilirubin	1.9	5.4	0	0
Renal (creatinine)	0	0	0	0
Metabolic				
Hyponatremia	1.9	8.9	5.9	2.9
Hypokalemia	1.9	7.1	2.9	2.9
Fever	0	3.6	0	0
Dermatologic: erythema	3.7	5.4	0	2.9

National Cancer Institute of Canada (NCIC) Clinical Trials Group (EORTC/NCIC TMZ study) reported, in 2005, that RT + TMZ significantly prolonged the survival of GBM patients compared to RT alone [24]. The median PFS, OS, and 2-year survival for RT + TMZ were 6.9, 14.6 months, and 26.5 %, respectively [24]. Although our results compared favorably with the EORTC/NCIC TMZ study, the PFS of RT + ACNU alone for GBMs in our ITT population and in GBM subgroups in central pathology review were 6.2 and 5.1 months, shorter than those from the EORTC/NCIC TMZ study. Since more than half of the patients in our study underwent TMZ treatment following disease progression, it is possible that TMZ rescued these patients with progression after ACNU regimens and prolonged the survival of these patients.

The incidence of grade 3/4 hematologic AEs—such as leukopenia, neutropenia, and thrombocytopenia—were reported to be 5, 4, and 11 %, respectively, in adjuvant TMZ therapy in the EORTC/NCIC TMZ trial [24]. Compared to TMZ, even ACNU alone caused severe hematologic AEs in 40 % of the patients in our study, and most of those patients in both arms discontinued the treatment protocol due to AEs or patient refusal related to AEs. It is noteworthy that approximately 30 % of patients in both arms failed to start adjuvant chemotherapy. The low completion rate of our protocol might explain the lack of differences in PFS and OS between the arms. After 2 patients in arm B experienced PCP, prophylactic use of cotrimoxazole (trimethoprim–sulfamethoxazole) against PCP was recommended in this study and was found to be useful.

Radiation necrosis has been reported in 2.5–21 % of patients undergoing chemoradiotherapy against malignant gliomas [25]. This complication was observed in 2.7 % of the patients in our study, but was tolerable. "Pseudo-progression" is the phenomenon of transient early disease progression after treatment with chemoradiotherapy consisting of TMZ for GBM progressive and enhancing lesions, as shown on MRI images taken immediately after treatment [25]. No patients in our study were suspected of pseudo-progression within 6 months after beginning chemoradiotherapy.

In general, the difference in histological diagnosis for local versus central pathology review is a major problem in the conduct of clinical trials on gliomas [26]. In our study, the concordance of GBM and AA between local and central diagnosis was low, but nearly identical to previous reports. In the EORTC/NCIC TMZ trial, central pathology review was performed in 85 % of cases, which confirmed the diagnosis of GBM in 93 % of the reviewed cases; 3 % had AA or AOA. In the phase III study of RT versus RT + BCNU + dibromodulcitol (EORTC 26882), of the 193 cases of AA diagnosed by the local pathologist, 176 were reviewed by the central pathologist. At review, 61 patients (35 %) were diagnosed with AA, 13 (8 %) with AOA, 4 (2 %) with AO, 44 (25 %) with GBM, 41 (23 %) with low-grade gliomas, and 13 (7 %) with another diagnosis [27].

The WHO classification system reflects the prognoses depending on grade I–IV tumors, or astrocytic or oligodendroglial tumors. However, it is based on morphological descriptions and contains subjective elements; thus, inter-observer variation occurs. The boundaries between grades II, III, and IV in gliomas are unclear, and there is a trend toward a more frequent diagnosis of oligodendroglial tumors [28]. Central pathological review before inclusion of a patient into clinical study is ideal, but it is very difficult to complete for aggressive grade III/IV tumors. Even if central review before enrollment is difficult in a multi-institutional setting, it is indispensable to perform post hoc central review at least in order to appropriately interpret the results of clinical studies of gliomas. A consensus meeting might also be useful before commencing clinical studies in order to gain concordance between local and central diagnoses. More objective classification of tumors based on

genotype, such the IDH1/2 mutation or 1p/19q codeletion, should be included in at least the stratification factor and subgroup analysis.

Conclusions

No significant differences in OS or PFS were found between ACNU alone and ACNU + PCZ in either AA or GBM. We found that ACNU + PCZ treatment was more toxic in our treatment schedule. Therefore, we conclude that the addition of PCZ to ACNU was not beneficial for newly diagnosed, high-grade astrocytomas as compared to ACNU alone. Considering the greater number of AEs associated with ACNU regimens, RT + TMZ should serve as a standard therapeutic regimen in the treatment of newly diagnosed AA and GBM.

Acknowledgments We thank all the members of the JCOG Brain Tumor Study Group and the staff of the JCOG Data Center. We appreciate Dr. Nobuaki Funata and Dr. Toru Iwaki for pathological review, Dr. Satoshi Ishikura for quality assurance of radiation therapy, and Dr. Hiroshi Katayama, Dr. Kenichi Nakamura, and Mr. Hidenobu Yamada for review of the manuscript. This work was supported in part by the National Cancer Center Research and Development Fund (23A-16 and 23A-20), the Health and Labour Sciences Research Grants (H14-032, H15-025, H16-005, H17-005), and the Grant-in Aid for Cancer Research (20S-4, 20S-6) from the Ministry of Health, Labour and Welfare.

Conflict of interest The authors declare that they have no conflict of interest.

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Brain Tumor Pathol (2012) 29:192–200
DOI 10.1007/s10014-012-0084-2

ORIGINAL ARTICLE

Pathological findings and prognostic factors in recurrent glioblastomas

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Received: 28 October 2011 / Accepted: 20 January 2012 / Published online: 14 February 2012

Abstract Glioblastomas, which are the most common primary intracranial tumor, are associated with the poorest survival time, which is typically 1–2 years. Age at initial diagnosis, Karnofsky performance score, and O^6-methylguanine DNA-methyltransferase (*MGMT*) promoter methylation status are the most well-documented predictors of survival in patients with newly diagnosed glioblastoma. Few studies have examined prognostic factors in patients with recurrent glioblastomas. At relapse, the pathological features of glioblastomas are affected by tumor regrowth and the influence of chemoradiotherapy during the initial treatment. Morphological transformations at recurrence include quantitative changes in tumor cells, such as the presence of giant cells and gemistocytic cell formation, radiation necrosis, and vascular structural changes. Therefore, we should carefully examine pathological findings at recurrence. In this report, we analyzed *MGMT* promoter status, the MIB-1 index, and the pathology of tumor samples at the first (primary tumor) and second (recurrent tumor) surgeries and clarified prognostic factors in patients with recurrent cases. In the multivariate analysis, we showed that MIB-1 indexes at the time of the second surgery ($p = 0.004$) persisted as a significant independent prognostic factor in survival of patients with recurrent glioblastoma.

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Keywords Recurrent glioblastoma · MIB-1 index · *MGMT* promoter methylation status · Prognostic factor

Introduction

Temozolomide (TMZ) is the standard therapy for patients with glioblastomas [1]. A recent study showed an improvement in median survival time (MST) from 12.1 to 14.6 months with the addition of concurrent TMZ to the previous standard therapy of surgery and radiotherapy in patients with glioblastomas [1]. Age at diagnosis, Karnofsky performance score (KPS), extent of surgical resection, and *MGMT* promoter methylation status have been well-documented prognostic factors of survival in patients with newly diagnosed glioblastomas [2–6]. Only a few studies have reported prognostic factors in patients with recurrent glioblastomas. The initial histology of the glioblastoma, increased patient age, KPS <80, and corticosteroid use have been reported to be poor prognostic factors for survival in patients with recurrent gliomas [7]. However, the prognostic factors of recurrent glioblastomas remain unclear. Higher MIB-1 indexes of gliomas have been demonstrated to correlate well with poorer survival time [4, 8, 9]. However, MIB-1 indexes of glioblastomas at the first surgery do not predict overall survival or the response to adjuvant therapy as an independent risk factor [10], and the significance of MIB-1 indexes in glioblastomas remains unclear. Methylated O^6-methylguanine DNA-methyltransferase (*MGMT*) promoter status could serve as a good prognostic factor for glioblastomas [11]. Variations in *MGMT* promoter methylation can occur within the same tumor after treatment [12, 13]. However, the prognostic value of variations in *MGMT* promoter methylation before and after chemoradiotherapy has been estimated in only a

few reports [14]. Giant cells, gemistocytic cell formation, and coagulation necrosis are often found in recurrent gliomas, and these findings suggest the presence of degenerative changes in tumor cells that are caused by hypoxia, irradiation, and chemotherapy [15]. Therefore, we also estimated the degenerative changes of tumor cells that are influenced by chemoradiotherapy in order to determine whether these degenerative changes may be a prognostic factor. Pathological features of glioblastomas at recurrence are affected by tumor regrowth and the influences of irradiation and chemotherapy during the initial treatment. Morphological transformations at recurrence include quantitative changes of tumor cells, radiation necrosis, and vascular structural changes [15–17]. Therefore, it is very difficult to estimate pathological findings at recurrence. In this study, we analyzed a number of prognostic factors, including MIB-1 indexes, methylation statuses of the *MGMT* promoter, and pathological findings in patients with recurrent glioblastomas.

Materials and methods

Patient and tissue collections

One hundred eighty-nine patients with glioblastoma were treated from 1996 to 2010 at our institute. Thirty-two patients (16.9%) were diagnosed initially with glioblastomas from 1996 to March 2010 and underwent second surgical resections for recurrence in the National Cancer Center Hospital. The recurrent surgical cases did not include any case of glioblastoma with oligodendroglial component (GBMO). Those patients underwent surgery twice or more during the treatment period of 1996–2010. They underwent initial surgeries, followed by chemoradiotherapy with nimustine hydrochloride (ACNU) or TMZ. Tumor samples were analyzed from primary and recurrent resected tumors; however, not all primary tumor samples resected in other hospitals were obtained. We only evaluated tumor samples with sufficient specimens for immunohistochemistry and DNA extraction. The MIB-1 index and *MGMT* promoter methylation status of the tumor samples from the first (primary tumor) and second (recurrent tumor) surgeries were determined. The presence of degenerative changes in the tumors, including pseudopalisading necrosis, coagulation necrosis, gemistocytic cells, and giant cells, was observed. The internal review board of the National Cancer Center approved this study. We defined the first progression-free survival (PFS) time as the time from the first operation to the first recurrence, and the second PFS was defined as the time from the second operation to the second recurrence. Detailed information on all 32 patients is listed in Table 1.

Table 1 Characteristics of patients with recurrent glioblastoma

Characteristic	Number of patients	Percent
Sex		
Male	20	62.5
Female	12	37.5
Age (years)		
Median	57	
Range	19–71	
Extent of removal at the first surgery		
Total removal	11	34.4
Subtotal removal	5	15.6
Partial removal	11	34.4
Biopsy	5	15.6
Extent of removal at the second surgery		
Total removal	5	15.6
Subtotal removal	5	15.6
Partial removal	20	62.5
Biopsy	2	6.3
MIB-1 index at the first surgery (%)		
Median	22.5	
Range	6.8–90.0	
MIB-1 index at the second surgery (%)		
Median	13.2	
Range	0.6–85.7	
MGMT promoter status at the first surgery		
Methylated	6	31.6
Unmethylated	13	68.4
MGMT promoter status at the second surgery		
Methylated	5	21.7
Unmethylated	18	78.3
Initial chemotherapy		
ACNU	20	62.5
TMZ	12	37.5
First PFS (months)		
Median	6.2	
Range	2.7–47.1	
Second PFS (months)		
Median	6.9	
Range	0.6–68.3	
Overall survival (months)		
Median	19.6	
Range	7.8–72.2	

ACNU nimustine hydrochloride, *TMZ* temozolomide, *PFS* progression-free survival

Histopathological analysis

Surgical specimens were fixed in 10% formalin and embedded in paraffin. Hematoxylin-and-eosin (H&E)-stained specimens were examined to determine histological tumor type. Degenerative changes, including pseudopalisading necrosis,

coagulation necrosis, gemistocytic cells, and giant cells, were also examined in H&E-stained specimens from the second operation. Multiple serial sections were subjected to immunohistochemical analyses in order to determine local staining. Furthermore, tissue sections were subjected to 15 min of microwave heating to activate antigens in a retrieval solution composed of 0.1 mol/L sodium citrate (pH 6). This was followed with immunostaining of the specimens with the streptavidin–biotin–peroxidase complex method (Vectastain; Vector Laboratories, Inc., Burlingame, CA, USA). Human monoclonal antibodies were used that recognize MIB-1 (Dako, Tokyo, Japan). Positive immunostaining was demonstrated with diaminobenzidine reactions, and slides were subsequently counterstained with hematoxylin, dehydrated, cleared, and mounted. Cell counting was performed with the aid of a light microscope (Olympus Corporation, Tokyo, Japan) at a magnification of 400×. At least 200 tumor cells were counted, and data consisted of the mean of the counts from three different locations within the specimen. MIB-1-stained cells were also counted and the percentage calculated within the observed field as the MIB-1 index.

Extraction of nucleic acids

Tumor samples were immediately frozen in liquid nitrogen and stored at −80°C. From each patient, a peripheral blood sample was drawn and stored at −80°C. Total DNA was extracted from either frozen tissue samples or paraffin-embedded specimens and each patient's blood with a DNeasy Blood & Tissue Kit (QIAGEN Sciences, Germantown, MD, USA), according to the manufacturer's protocol.

MGMT promoter methylation analysis

MGMT promoter methylation status was determined by methylation-specific polymerase chain reaction (PCR) (MSP). Tumor DNA was subjected to bisulfite treatment overnight at 50°C and then purified using an EZ DNA Methylation Kit (Zymo Research Corporation, Irvine, CA, USA), according to the manufacturer's protocol. Tumor DNA obtained from paraffin-embedded specimens was amplified with the use of the first PCR using the following primers: 5′-GGATATGTTGGGATAGTT-3′ and 5′-CCA AAAACCCCAAACCC-3′ and then subjected to MSP (two-step approach) [11, 18]. Thermocycling conditions consisted of 5 min at 95°C, 35 cycles of 45 s at 95°C, 30 s at 52°C, and 50 s at 72°C. Tumor DNA obtained from frozen tissues was directly subjected to MSP. Primer sequences used to amplify sequences from the methylated or unmethylated *MGMT* promoter were 5′-TTTCGACG TTCGTAGGTTTTCGC-3′ (M-MGMT-F) and 5′-GCAC TCTTCCGAAAACGAAACG-3′ (M-MGMT-R) or 5′-TT TGTGTTTTGATGTTTGTAGGTTTTTGT-3′ (U-MGMT-F) and 5′-AACTCCACACTCTTCCAAAAACAAAACA-3′ (U-MGMT-R). Thermocycling conditions for the methylated *MGMT* promoter consisted of 5 min at 95°C, 35 cycles of 45 s at 95°C, 30 s at 67°C, and 50 s at 72°C. Thermocycling conditions for the unmethylated *MGMT* promoter consisted of 5 min at 95°C, 35 cycles of 45 s at 95°C, 30 s at 64°C, and 50 s at 72°C. PCR products were separated on 2% agarose gels.

Statistical analysis

A multivariate analysis with the Cox model, which was used to assess truly independent prognostic factors, was performed only for variables for which p values <0.1 were obtained in the univariate analysis (JMP ver 8, Tokyo, Japan).

Results

Overall and progression-free survival of glioblastoma patients

Overall survival and PFS of 189 patients with newly diagnosed glioblastomas who were treated from 1996 to 2010 at our institute were 15.1 and 7.7 months, respectively (M:F = 121:68; median age 60.0 years). The MST of patients treated with initial chemoradiotherapy with ACNU ($n = 79$), TMZ ($n = 91$), and radiation only ($n = 19$) were 14.8, 16.2, and 6.6 months, respectively. There was no significant difference in MST after initial chemoradiotherapy with ACNU and TMZ ($p = 0.32$). The PFS of patients treated with initial chemoradiotherapy with TMZ is significantly longer than those treated with ACNU (10.0 and 5.3 months, $p < 0.01$). Patients who had undergone surgery twice or more ($n = 35$; MST 21.0 months) showed longer overall survival time than those who had undergone surgery only once ($n = 154$; MST 12.9 months; $p = 0.008$). The MST of patients in the total or subtotal resection group ($n = 69$; median age 62.0 years) and that of patients in the partial or biopsy group ($n = 120$; median age 60.0 years) who underwent a first surgery was 17.6 and 13.4 months, respectively ($p = 0.03$). Thirty-two patients with glioblastoma relapsed and underwent a second surgery at the first recurrence. Eight of 32 patients underwent a third operation at the second recurrence. Only one patient underwent a fourth operation at the third recurrence. The first median PFS was 6.2 months and the second 6.9 months. In the univariate analysis (Table 2), patients ≤50 years had a longer survival time than those who were 50 years old ($p = 0.05$). MST of patients ≤50 years was 31.2 months and of those who were 50 years was 16.6 months.