dence is available.[24] However, a recent report suggested that early surgical treatment of LGG is associated with better survival than observation, and more aggressive treatment thus appears warranted for optimal treatment of LGG.[8] Although the role of surgical resection for LGG remains controversial,[9,24] emerging evidences suggests EOR is important for survival in patients with LGG.[12,23] In our experience of 153 cases, EOR was strongly correlated with prognosis in patients with LGG. Both OS and PFS in our study were consistent with other reports that have shown benefits of surgical resection for patient survival.[12,23]

Given that LGG will eventually progress or undergo malignant transformation, reducing the number of tumor cells as far as possible to prevent progression or malignant transformation of the tumor appears reasonable. One of the limitations of previous studies that have not shown the benefit of surgical resection is that the EOR was based on the intraoperative interpretation of the surgeon or nonquantitative estimates.[17,24] Meticulous estimate of pre- and postoperative tumor volume and EOR using volumetric calculation is thus critical for accurate evaluation of the importance of EOR for patient prognosis.[7,23] Which sequence of MR imaging is most suitable for volumetric analysis of EOR remains unknown. Fluid-attenuated inversion recovery (FLAIR) imaging seems to provide better definition of spread of the tumor than T_2-weighted imaging. However, intraoperative MR imaging in our institute does not give adequate quality of FLAIR imaging, so we have been using T_2-weighted imaging for the volumetric analysis.

The boundaries of the tumor and normal brain tissue in LGG are difficult to distinguish compared to its malignant counterparts, and gross total resection of LGG is thus difficult. Use of intraoperative MR imaging can drastically reduce the amount of residual tumor.[13,22] Safe and maximum resection of LGG located in eloquent areas is difficult and intraoperative cortical mapping under awake craniotomy is useful.[2-4] Our department has introduced intraoperative MR imaging and has experienced more than 900 glioma surgeries since 2000.[13] We have also performed more than 250 cases of intraoperative functional mapping with awake craniotomy for gliomas located in eloquent areas to achieve both maximum resection and preservation of neurological function. Use of intraoperative MR imaging in the setting of awake craniotomy enables safe and maximum resection of LGG.[16] Thus information-guided surgery, integrating anatomical, functional, and histopathological data, permits the surgeon to achieve maximum resection with minimum risk of neurological deficit.[6,14,26]

Detailed characterization of resected tumor tissue during surgery is important in the accurate diagnosis of tumor border and maximum resection. We showed that EOR is strongly correlated with both OS and PFS in patients with astrocytoma. In other words, the prognosis for patients with astrocytoma who underwent partial resection was significantly worse. Furthermore, our analysis showed that neither radiation nor nitrosourea-based chemotherapy showed any survival benefit in patients with astrocytoma (data not shown). Maximum resection will be thus critical for successful treatment for patients with astrocytoma. Treatment for patients with astrocytoma in which surgery ended in partial removal is therefore difficult. A randomized study to evaluate the role of postsurgical treatments for partially resected astrocytoma will thus be necessary.

Interestingly, EOR did not affect PFS in patients with oligodendroglioma in our study, possibly because oligodendroglioma infiltrates beyond MR imaging-defined abnormalities.[15] Currently our strategy for oligodendroglioma is gross total resection of the high intensity lesion on T_2-weighted MR imaging, but more extensive resection may be required to prevent progression of oligodendroglioma.[15] However, concluding that surgical resection does not contribute patient survival would be premature, because patients with oligodendroglioma survive longer and OS may differ after longer follow-up periods. In fact, this study included relatively few death events for patients with oligodendroglioma. Careful long-term assessment of OS may be important to assess the importance of EOR in oligodendroglioma. This is the first report that showed different effects of EOR on patient PFS between tumor subtypes.

Given the present results, we have updated our therapeutic strategy for LGG stratified by EOR and tumor subtype (Fig. 4). Our findings suggest that EOR $\geq 90\%$ is strongly correlated with the prognosis of astrocytoma, and the patient can be observed carefully without postsurgical treatment regardless of tumor subtype. If surgery resulted in EOR $< 90\%$, patients with astrocytoma will require second-look surgery, whereas patients with oligodendroglioma or oligoastrocytoma, which are sensitive to chemotherapy, will be treated with chemotherapy. We are now collecting genomic information including co-deletion of chromosome arms 1p and 19q, and mutation of IDH1 and IDH2. Integration of the genetic information into our updated strategy for the treatment of LGG will be necessary in the future. In conclusion, given the different prognosis and effects of EOR between tumor subtypes, treatment of

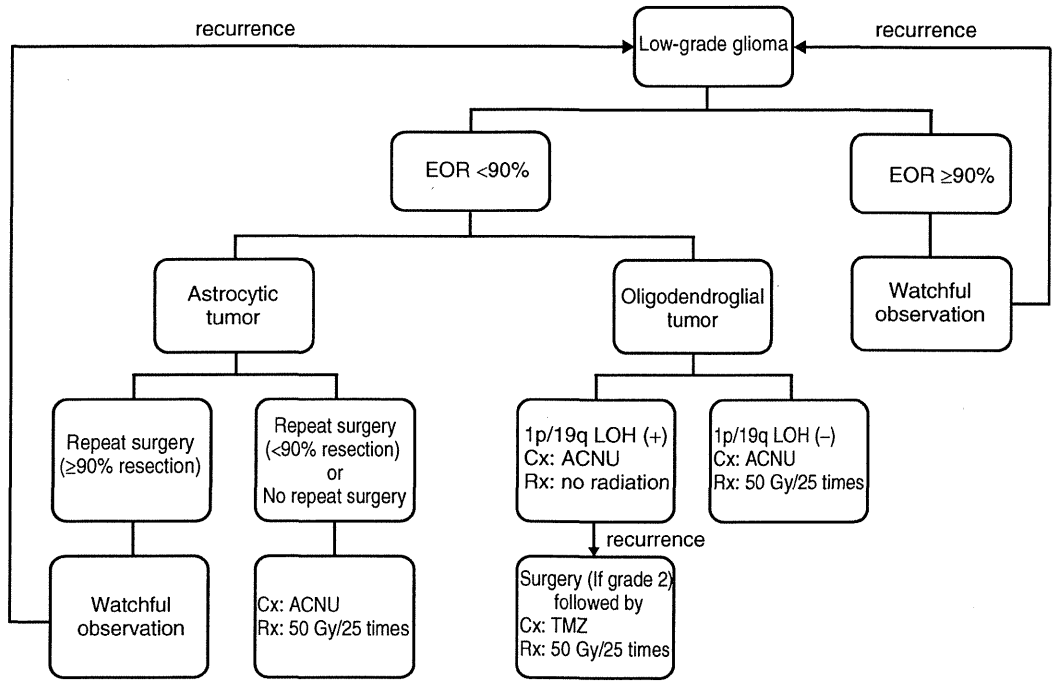

Fig. 4 Therapeutic strategy for low-grade glioma stratified by extent of resection (EOR) and tumor subtype. If the EOR is ≥90%, patients can be observed carefully without postsurgical treatment regardless of tumor subtype. If surgery resulted in EOR <90%, patients with diffuse astrocytoma will require second-look surgery, whereas those with oligodendroglioma or oligoastrocytoma, which are sensitive to chemotherapy, will be treated with chemotherapy. Cx: chemotherapy, LOH: loss of heterozygosity, Rx: radiation, TMZ: temozolomide.

LGG should be stratified by EOR and tumor subtype.

Conflicts of Interest Disclosure

The authors declare that they do not have any conflict of interests. All authors who are members of The Japan Neurosurgical Society (JNS) have registered online Self-reported COI Disclosure Statement Forms through the website for JNS members.

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Phase I/IIa trial of autologous formalin-fixed tumor vaccine concomitant with fractionated radiotherapy for newly diagnosed glioblastoma

Clinical article

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Object. The objective of the present study was analysis of results of the prospective clinical trial directed toward the evaluation of therapeutic efficacy of the administration of autologous formalin-fixed tumor vaccine (AFTV) concomitant with fractionated radiotherapy in cases of newly diagnosed glioblastoma multiforme.

Methods. Twenty-four patients were enrolled into the clinical trial, while 2 cases were excluded from the final analysis of results. The treatment protocol included aggressive tumor resection, fractionated radiotherapy up to a total dose of 60 Gy, and 3 concomitant courses of AFTV administered with an interval of one week at the late stage of irradiation. Two delayed-type hypersensitivity (DTH) tests were done—one 48 hours before the initial course of vaccination (DTH-1) and one 2 weeks after the third (DTH-2). All but one of the patients received salvage therapy at the time of tumor progression. The defined primary end point was overall survival; secondary end points were progression-free survival and safety of concomitant treatment.

Results. The median duration of overall survival was 19.8 months (95% CI 13.8–31.3 months). The actuarial 2-year survival rate was 40%. The median duration of progression-free survival was 7.6 months (95% CI 4.3–13.6 months). Overall survival showed a statistically significant association with recursive partitioning analysis class ($p < 0.05$); progression-free survival showed a statistically significant association with p53 staining index ($p < 0.05$) and size of DTH-2 response ($p < 0.001$). AFTV injection concomitant with fractionated radiotherapy was well tolerated by all patients and in no case did treatment-related adverse effects exceed Grade 1 toxicity; adverse effects were limited to local erythema, induration, and swelling at the site of injection.

Conclusions. The results of this study demonstrate that AFTV treatment concomitant with fractionated radiotherapy may be effective in patients with newly diagnosed glioblastoma. Further clinical testing is warranted.
(DOI: 10.3171/2011.4.JNS10377)

Key Words • **brain tumor vaccine therapy** • **glioblastoma** • **autologous formalin-fixed tumor vaccine** • **oncology**

Despite recent advances in aggressive resection combined with radiochemotherapy, management of glioblastoma represents a significant clinical challenge. More than three-quarters of patients with this

This article contains some figures that are displayed in color online but in black and white in the print edition.

Abbreviations used in this paper: AFTV = autologous formalin-fixed tumor vaccine; CEA = carcinoembryonic antigen; DTH = delayed-type hypersensitivity; EGFR = epidermal growth factor receptor: GBM = glioblastoma multiforme; KPS = Karnofsky Performance Scale; MHC = major histocompatibility complex; MRC = Medical Research Council; OS = overall survival; PFS = progression-free survival; RPA = recursive partitioning analysis.

tumor die within 2 years after surgery and less than 10% survive for 5 years.[2,19,21,22] This dismal prognosis continues to stimulate the search for additional treatment options.

Recently, there has been a growing interest in therapeutic modalities based on tumor-specific immune reactions, which have a potentially high benefit-to-risk ratio. Preliminary clinical trials had revealed favorable results for immunotherapy of recurrent malignant glioma with ex vivo expanded autologous tumor-specific cytotoxic T lymphocytes.[25,26] The usefulness of this approach is, however, limited due to the necessity of propagating a large quantity of autologous tumor cells and time-consuming tedious culturing of the cytotoxic T lymphocytes; neither of these processes is always successful. An alternative approach was proposed by Ohno and colleagues, who developed use of formalin-fixed sections instead of live target tumor cells for ex vivo cytotoxic T lymphocyte induction[9,10] and later formulated the autologous formalin-fixed tumor vaccine (AFTV) for in vivo induction of killer lymphocytes.[13,14] A Phase II randomized clinical trial showed that AFTV prevents recurrence of hepatocellular carcinoma after surgery,[8] and another pilot study revealed its therapeutic effectiveness associated with absence of severe treatment-related complications in cases of recurrent and residual GBM.[6] Therefore, the present prospective clinical trial was initiated for evaluation of the therapeutic efficacy of AFTV concomitant with fractionated radiotherapy in patients with newly diagnosed GBM.

Methods

Study Design

A prospective clinical trial of AFTV concomitant with fractionated radiotherapy for management of newly diagnosed GBM was conducted by the Association of Cancer Vaccine Therapy in 2 participating hospitals, Tokyo Women's Medical University Hospital (Tokyo, Japan) and the Tsukuba University Hospital (Ibaragi, Japan). According to objectives focused on preliminary evaluation of the therapeutic efficacy and safety of treatment, the trial was designated as Phase I/IIa, and dose escalation was not planned. The study design and treatment protocol were approved by the ethics committees of both institutions and registered in the University Hospital Medical Information Network (UMIN) clinical trials registry (identification no. C000000002, Tokyo). Eligibility and exclusion criteria for patient enrollment are presented in Table 1. Written informed consent for participation in the study was obtained in each individual case. The 2-year study period started on August 10, 2005, and enrollment of 25 patients was planned. Only 24 patients, however, were actually enrolled. The trial was developed and initiated before temozolomide (Temodal, Schering-Plough) treatment (150–200 mg/m^2 daily for 5 days every 28 days) was approved by the Japanese government for malignant gliomas (September 15, 2006). When temozolomide treatment was approved, the concept of the study was reconsidered by the ethics committees of both participating institutions, and it was decided to continue the trial within the designated time period (up to August 9, 2007), with additional information on possible chemotherapy with temozolomide being provided for each patient before enrollment into the study.

TABLE 1: Eligibility and exclusion criteria*

Eligibility Criteria
age: 16–75 yrs
newly diagnosed GBM w/ histopathological confirmation of the diagnosis
manifestation of the disease w/ typical neurological symptoms
maximum possible resection of the tumor (radiologically complete removal or subtotal removal leaving the residual neoplasm w/in the vital, functionally important brain areas)
availability of at least 1.5 g of neoplastic tissue for AFTV preparation
possibility for in-house AFTV preparation & administration
completed course of postop FRT w/ a cumulative dose of 60 Gy
KPS score ≥60 before initiation of FRT
possibility of regular follow-up evaluation
Exclusion Criteria
treatment w/ glucocorticoids or antitumor chemotherapy
presence of intracranial hypertension at time of scheduled AFTV treatment
suppressed hematological function according to the Common Toxicity Criteria version 2 (National Cancer Institute) or absolute WBC count ≤2000/mm^3
decompensated function of internal organs
presence of malignant tumor other than GBM
planned or existing pregnancy
enrollment in another clinical trial w/in the 6 mos preceding the present study
ineligibility as judged by the principal investigator of the participating institution (for example, due to anticipation of problems w/ regular follow-up evaluations caused by distant address or socioeconomic issues)

* FRT = fractionated radiotherapy; WBC = white blood cell.

After confirmation of the histopathological diagnosis of GBM following resection of newly diagnosed parenchymal brain tumor, the eligible patients who provided their agreement to participate in the study were scheduled for fractionated radiotherapy and concomitant treatment with AFTV according to the standard protocol (Fig. 1). In each case the histopathological diagnosis was independently confirmed in the Japan Brain Tumor Reference Center (Y.N.) in Gunma University (Maebashi, Japan) according to the current WHO criteria, using paraffin-embedded tissue sections stained with H & E. Additional immunohistochemical analysis included evaluation of the positive cells using monoclonal antibodies for MIB-1 (Dako), p53 (Dako), and MHC Class I (Hokudo Co.). The corresponding staining indices were calculated as an average number of positive cells in the best-stained tumor areas (up to 5) with a total amount of cells not less than 1000. The MIB-1 and p53 indices were expressed as percentages, whereas MHC Class I expression was graded as 0 (absence of staining), + (up to 25% of cells stained), ++ (25%–50% of cells stained), or +++ (more than 50% of cells stained).[1,6]

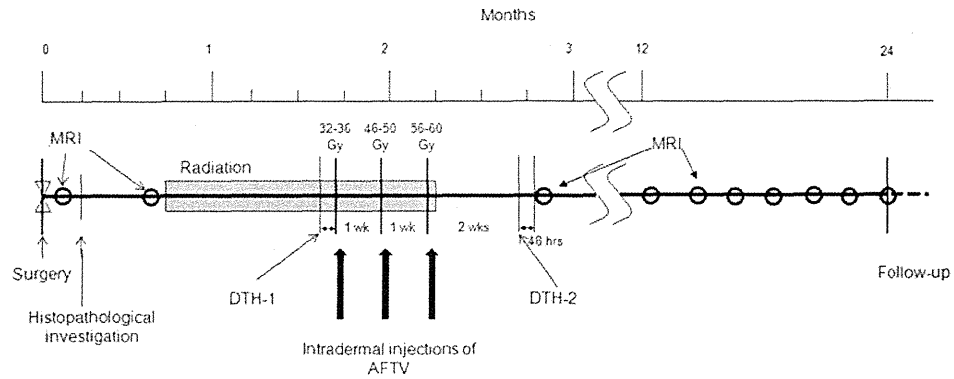

Fig. 1. Scheme of prospective clinical protocol for AFTV treatment in patients with newly diagnosed glioblastoma.

The baseline clinical investigations at the time of enrollment into the trial included physical examination with evaluation of KPS scores and determination of the MRC neurological functional grade, blood and urine tests, electrocardiogram, chest radiograph, and brain MR imaging obtained initially within 3 days after surgery and additionally just before the first fractionated radiotherapy session.

Preparation of AFTV

Autologous formalin-fixed tumor vaccine was prepared using autologous formalin-fixed GBM tissue according to an established standard operating procedure as described previously.[6] In short, the formalin-fixed histologically confirmed viable neoplastic tissue was initially thoroughly fragmented and centrifuged at 11,100 G for 5 minutes; 0.1 ml of alcohol extract prepared from 1.2 mg of freeze-dried Bacillus-Calmette Guérin (BCG) vaccine (Japan BCG Laboratory) was added to 0.22 ml of the packed tumor tissue pellet obtained after centrifugation; the pellet was washed with saline; and final concentration of the tissue fragments was adjusted to 20% (v/v, packed volume) suspended in 1 ml saline, which also contained 250 ng of tuberculin microparticles and 250 ng of soluble tuberculin (Japan BCG Laboratory).

Treatment Protocol

Fractionated radiotherapy was started within 2–3 weeks after resection of the neoplasm and included focal irradiation of the tumor cavity or residual lesion including 2 cm of perifocal margin with 2 Gy per fraction up to a total dose of 60 Gy. When the radiation dose reached 32–36 Gy the concomitant AFTV treatment was initiated. The AFTV treatment consisted of 3 courses of vaccination performed at intervals of 1 week.[6] Each course consisted of 5 local intradermal injections of 0.2 ml of AFTV per site in the upper arm.

Two delayed-type hypersensitivity (DTH) tests[6] were performed 48 hours before the first course of vaccination (DTH-1) and 2 weeks after the third course (DTH-2). For these tests, fixed autologous tissue fragments (10% v/v suspended in 0.1 ml of saline in the absence of immune adjuvant) were injected intradermally into the forearm, and response was evaluated by diameter of the local erythema and induration.

Clinical Characteristics of the Enrolled Patients

From August 10, 2005, to August 7, 2007, 24 patients with newly diagnosed GBM were enrolled into this prospective clinical trial (19 in Tokyo Women's Medical University Hospital and 5 in Tsukuba University Hospital). Two patients, however, were subsequently excluded. In Case 12, 2 separate brain tumors were present (one in the left parietotemporal lobe and one in the right temporal lobe), but only one was irradiated. The patient was excluded from final analysis because it was thought that progression of the nonirradiated neoplasm could influence survival, defined as the trial end point (see below). In Case 20, after initially agreeing to participate in the study, the patient refused scheduled AFTV injection during the course of radiotherapy. Clinical characteristics of the remaining 22 patients are presented in Table 2.

The group of 22 patients included 15 men and 7 women. Their age varied from 18 to 70 years (median 58 years). The preoperative KPS score was 90 or 100 in 14 patients, 70 or 80 in 3, and less than 70 in 5 (median 90). By the time of initiation of fractionated radiotherapy all patients had KPS scores of at least 70. With respect to RPA classification,[3] 7 patients (32%) were assigned Class III, 8 (36%) Class IV, and 7 (32%) Class V. Before surgery the maximum tumor diameter varied from 15 to 103 mm (median 50 mm). In 16 cases (73%) the resection of the neoplasm was considered total (98% or more of the contrast-enhancing lesion), and in 6 (27%) partial. In all cases the histopathological diagnosis of GBM was independently confirmed in the Japan Brain Tumor Reference Center. The MIB-1 staining index varied from 7.7% to 66.8% (median 29.1%). The p53 staining index varied from 0 to 85% (median 8.5%). The grades of MHC Class I expression were 0 in 2 cases, + in 8 cases, ++ in 7 cases, and +++ in 5 cases. There were no evident differences between the patient cohorts treated in the Tokyo Women's Medical University Hospital and the Tsukuba University Hospital, other than typically more aggressive tumor resection in the former institution due to the use of intraoperative MR imaging.

Follow-Up

Follow-up investigations were performed 2 weeks (14 ± 2 days) after completion of radiotherapy and every 2 months thereafter. Follow-up examinations included

TABLE 2: Characteristics of 22 patients*

Case No.	Age (yrs), Sex	Tumor Location	Preop Tumor Size (mm)	Preop KPS Score	RPA Class	Extent of Resection	MIB-1 Staining Index (%)	p53 Staining Index (%)	MHC Class I Expression Grade	Size of DTH-2 Response (mm)	OS (mos)	PFS (mos)
1	36, F	lt F-I	70	90	III	partial	45.5	59	++	1	42.0+	2.2
2	59, M	lt F & lt F	40	90	IV	total	26.4	37	+	10	6.1	3.3
3	34, M	bilat F	47	100	III	partial	35.9	85	+++	10	16.4	2.5
4	58, F	rt T	35	90	IV	partial	9.4	3	+++	8	14.4	2.3
5	66, M	lt P-O	50	80	IV	partial	52.6	46	+++	9	8.4	4.3
6	65, M	lt F	32	90	IV	total	13.7	0	+++	18	39.0	25.6
7	48, M	lt I	15	100	III	total	20.3	1	+	10	31.3	11.1
8	18, M	lt I	45	100	III	total	39.6	0	+++	15	41.6+	6.4
9	68, F	rt T & rt P	75	30	V	partial	34.4	0	++	8	9.5	6.6
10	64, M	rt O	60	90	V	total	26.9	3	+	11	6.4	4.5
11	58, M	rt O-T	103	50	V	total	30.4	1	+	12	26.9	14.0
13	70, M	rt F	35	70	V	total	31.0	78	+	4	12.7	3.8
14	54, F	lt T	37	50	V	total	24.9	66	+	35	22.6	13.9
15	41, M	lt F-P	50	70	IV	total	33.7	65	0	12	21.4	8.7
16	48, F	lt O	55	60	V	total	25.0	0	+	12	18.2	7.1
17	43, M	lt T	53	90	III	total	66.8	36	0	20	15.9	8.3
18	58, F	lt F	69	50	V	total	43.1	6	++	47	14.5	7.6
19	65, M	lt P	46	90	IV	total	17.5	11	++	19	13.8	5.0
21	32, M	lt F	65	100	III	partial	35.0	18	++	16	23.6+	18.5
22	61, M	lt T	52	90	IV	total	27.7	13	++	10	23.3+	13.6
23	60, F	lt T	48	100	IV	total	10.5	1	++	14	22.7+	14.4
24	26, M	rt F-T	57	100	III	total	7.7	5	+	20	20.3+	16.8

* The patients in cases 1–5 were treated at the Tsukuba University Hospital. All other patients were treated at Tokyo Women's Medical University Hospital. Multicentric gliomas were present in Cases 2 and 9. Cases 12 and 20 are not included due to violation of the study protocol (see text for details). The patient in Case 2 had 2 lesions, both in the left frontal lobe. Abbreviations: F = frontal; I = insular; O = occipital; P = parietal; T = temporal.

physical examination with evaluation of KPS score and determination of MRC neurological functional grade, blood and urine tests, and brain MR imaging. Adverse effects of treatment were graded according to the National Cancer Institute Common Toxicity Criteria version 2.0.[24]

No additional treatment was performed until tumor progression, which was defined as a 25% or greater increase in the volume of the contrast-enhanced lesion or appearance of new brain lesions.[11] At the time of neoplasm regrowth the patient was treated according to the preference of his or her doctor. In total, 21 of 22 patients underwent various types of salvage treatment (Fig. 2). In 10 cases (45%) at least 1 re-resection of the tumor was performed (with or without use of intraoperative photodynamic therapy). Chemotherapy with temozolomide (150–200 mg/m^2 daily for 5 days every 28 days) was administered in 20 patients, and 15 of them received at least 3 cycles of treatment. Among other therapies, proton-beam irradiation was performed in 1 case.

End Points and Statistical Analysis

The primary end point of the clinical trial was OS, defined as the time interval from the date of surgery to death from any cause. Secondary end points were PFS and safety of treatment. The following prespecified factors were analyzed for their association with OS and PFS: age, sex, tumor size before surgery, preoperative KPS score, RPA class, extent of tumor resection, MIB-1 staining index, p53 staining index, MHC Class I expression grade, and size of DTH-1 and DTH-2 response. Univariate analysis was performed using a log-rank test after construction of Kaplan-Meier survival curves. Continuous variables were dichotomized with regard to their median values. Factors that showed statistical significance were included in a Cox proportional hazard model for multivariate analysis. Differences were considered statistically significant if the 2-tailed p value was < 0.05.

Results

The length of follow-up varied from 6 to 42 months (median 19 months), and was 20 months for the last patient enrolled into the trial. At the time of data analysis, 6 patients (27%) were alive and 16 (73%) were dead.

Delayed-Type Hypersensitivity Test

No patient showed a positive response to the DTH-1 test, whereas response to the DTH-2 test varied in size from 1 to 47 mm (median 12 mm).

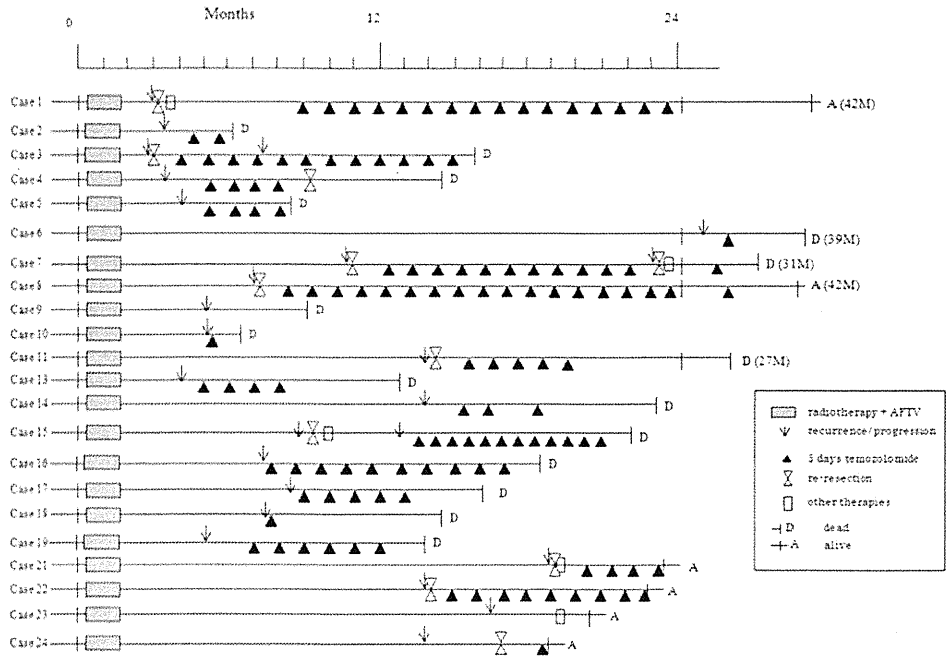

Fig. 2. Treatments applied after initial resection and outcome in each patient.

Overall Survival

The duration of OS varied from 6.1 to 42 months (median 19.8 months, 95% CI 13.8–31.3 months). The actuarial 2-year survival rate was 40% (Fig. 3).

The duration of OS showed a statistically significant association with RPA class. The median OS values in cases with RPA Class III, IV, and V, were 31.3, 21.4, and 14.5 months, respectively ($p < 0.05$).

In patients with a size DTH-2 response of 12 mm or larger, OS was longer than in those who had a response smaller than 12 mm; this difference, however, did not reach statistical significance (Fig. 4). The median OS values in these subgroups were 22.6 and 14.4 months, respectively ($p = 0.19$).

Other analyzed factors did not show statistically significant associations with OS.

Progression-Free Survival

The duration of PFS varied from 2.2 months to 25.6 months (median 7.6 months, 95% CI 4.3–13.6 months). It was significantly longer in cases in which the p53 staining index was lower than 8.5% (median PFS 7.5 months) than in cases in which the values were greater than 8.5% (median PFS 5.0 months, $p < 0.05$).

The duration of PFS in patients with a DTH-2 response of 12 mm or larger was significantly longer than in patients with a smaller DTH-2 response (Fig. 4). The median PFS in these subgroups was 13.9 months and 4.3 months, respectively ($p < 0.001$). The statistically significant difference in PFS was preserved when the cutoff level of DTH-2 response size was reduced to 10 mm (data not shown).

Other analyzed factors did not show statistically significant associations with PFS. Both the p53 staining index and size of DTH-2 response preserved their statistically significant associations with PFS in multivariate analysis.

Treatment Safety

Autologous formalin-fixed tumor vaccine treatment concomitant with fractionated radiotherapy was well tolerated by all patients. Vaccination did not result in any

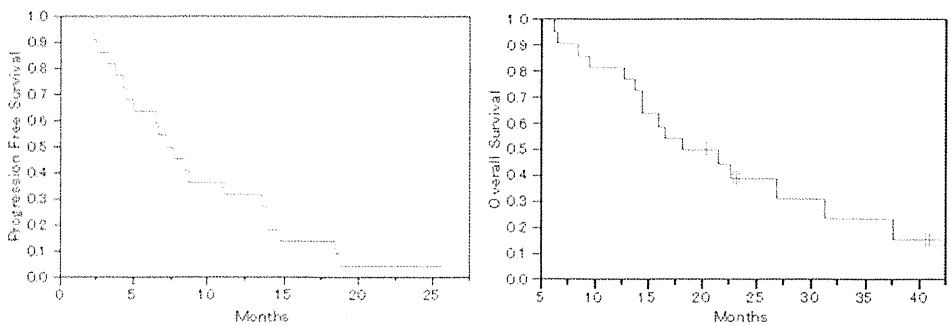

Fig. 3. Kaplan-Meier curves for PFS **(left)** and OS **(right)**. Censored observations are marked.

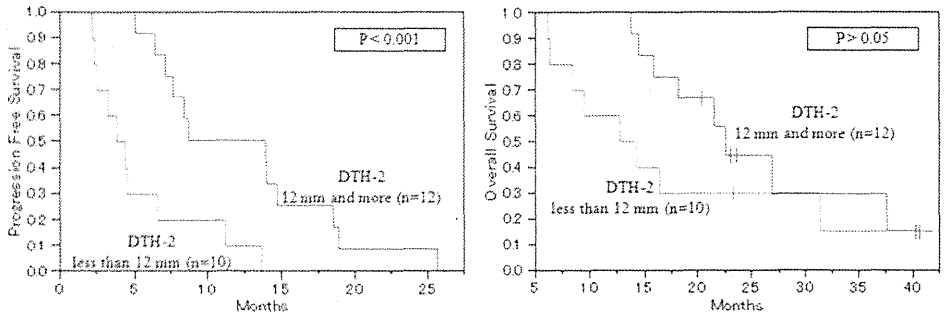

FIG. 4. Comparison of PFS **(left)** and OS **(right)** in subgroups of patients with different sizes of response to DTH testing after completion of vaccination (DTH-2). Censored observations are marked.

changes in KPS score. The treatment-related adverse effects consisted of local erythema, induration, and swelling at the injection sites and were observed in 21 of 22 patients, but in all cases these effects corresponded to Grade 1 toxicity.[24] No hematological toxicity—namely leukopenia, neutropenia, lymphopenia, thrombocytopenia, or anemia—was seen. No allergic dermatitis or anaphylaxis was observed, and there was no evidence of any autoimmune phenomena. Blood investigation before and after the vaccination did not detect significant abnormalities of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total bilirubin, total protein, lactate dehydrogenase, albumin, Na, K, Cl, glucose, or hemoglobin levels, or hematocrit or cell counts (red blood cells, white blood cells, platelets). In one patient (Case 1) a chronic subdural hematoma was revealed on the side contralateral to the craniotomy, but it was judged to be unrelated to AFTV treatment.

Discussion

The generally dismal prognosis associated with glioblastoma stimulates the search for novel treatment strategies. Several clinical trials have investigated the effectiveness and safety of vaccines in cases of recurrent malignant gliomas with or without concomitant chemotherapy,[4,15] or in patients with newly diagnosed tumors after completion of fractionated radiotherapy (without chemotherapy).[16,18,20] Nevertheless, to the best of our knowledge, there have been no data reported on the use of vaccine concomitant with fractionated radiotherapy alone for newly diagnosed glioblastoma.

Autologous formalin-fixed tumor vaccine is a novel, stable, and clinically durable vaccine that is simple to produce. In comparison with the promising newest types of peptide vaccines, such as EGFR variant III[17] and Wilms' tumor–1 (WT-1),[7] use of AFTV does not require preselection of patients according to expression of tumor-associated antigens. Its preparation is based on the fact that peptide tumor-associated antigens derived from fixed cells or proteins are formalin resistant; therefore, formalin-fixed and/or paraffin-embedded tumor cells/tissues may be used to generate effective antitumor immune cells.[13,14] Human leukocyte antigen-A2402–restricted CEA-specific cytotoxic T lymphocytes can be induced by culturing human peripheral blood mononuclear cells (PBMCs) with formalin-fixed autologous adhesive PBMCs loaded with CEA protein–bound latex beads, or can be generated using formalin-fixed adherent cells pulsed with 9- or 10-mer CEA-derived MHC Class I–presented tumor-associated antigens.[6,13] It was demonstrated that the activity and specificity of cytotoxic T lymphocytes generated by formalin-fixed tumor cells are comparable to those induced by continuously cultured live tumor cells.[9,10]

Autologous formalin-fixed tumor vaccine showed positive immunotherapeutic effects in experimental tumor models,[13] as well as in patients with hepatocellular carcinoma[8] and glioblastoma.[6] Ishikawa et al.[6] evaluated results of such treatment in 12 patients with either recurrent or residual glioblastoma. The AFTV was given as 3 five-site intradermal inoculations performed at weekly intervals. Response to DTH testing was evaluated before and after each vaccination. Of 12 tumors, 1 showed complete response, 1 showed partial response, 2 showed minor response, 1 showed stabilization of growth, and 7 progressed. The median duration of survival after initiation of AFTV treatment was 10.7 months, but 3 of 5 responders survived 20 months or longer. A low p53 staining index and high MHC Class I expression were associated with better survival. The treatment was well tolerated by all patients with only local erythema, induration, and low-grade fever being reported.[6]

Similarly, the present study demonstrates that a treatment protocol involving 3 courses of AFTV during the late stage of fractionated radiotherapy may be effective in patients with newly diagnosed glioblastoma. The median OS in this series was 19.8 months, whereas in the similar cohort treated with radiation alone during the EORTC 26981/22981–NCIC trial, it was 12.1 months.[21] The median OS in our patients with RPA Class V (14.5 months) was comparable to those in the best prognostic category of glioblastoma (RPA Class III) in the above-mentioned cohort (14.8 months).[21] Such results probably were not caused by salvage chemotherapy with temozolomide at the time of recurrence, because 70% of patients treated with radiotherapy alone during the EORTC 26981/22981–NCIC trial also received salvage temozolomide treatment.[12,21] Therefore, while compromise due to selection bias cannot be ruled out in our study, the effect of AFTV on the outcome can be strongly suspected.

It is important, that both the OS and PFS of our patients were associated with response to the DTH test, and

patients with a greater response to DTH-2 had statistically longer PFS. Because DTH response is strongly correlated with cell-mediated acquired immune response in vivo, it can be assumed that patients with a large DTH-2 response had developed activation of the cellular immune response against the autologous tumor cells. In agreement with the previous report,[6] PFS in our series was also associated with the p53 staining index of the tumor; therefore, it can be speculated, that a p53-related mechanism is somewhat involved in AFTV-induced cytotoxic T-lymphocyte activity.

Treatment with AFTV was not accompanied by significant associated morbidity in this study. In no patient did we identify reduction of KPS score or more than Grade 1 toxicity. The latter was limited to local erythema, induration, and swelling at the site of injection. While autoimmune encephalomyelitis represents a potentially serious complication of any type of glioma cell vaccine therapy,[27] no evidence of its development was observed in any of our patients.

The main limitation of our study is the omission of concomitant temozolomide chemotherapy during the course of postoperative fractionated radiotherapy, which is currently considered standard management of newly diagnosed glioblastoma.[5,12,21,22] In 2005, Stupp et al.[22] reported that such a treatment strategy has definite prognostic advantages and extends the median PFS from 5.0 to 6.9 months, extends the median OS from 12.1 months to 14.6 months, and increases the proportion of 2- and 5-year survivors from 10.4% to 26.5% and from 1.9% to 9.8%, respectively. However, our investigation was initiated before approval of such a radiochemotherapy protocol by the Japanese government (September 15, 2006). Therefore, taking into account the results presented herein, another clinical trial on the use of AFTV therapy during irradiation of newly diagnosed glioblastoma and concomitant chemotherapy with temozolomide is definitely needed. In fact, there is a theoretical possibility that additional vaccine therapy may enhance the therapeutic effectiveness of chemotherapy. Severe lymphopenia, especially depletion of $CD4^+$ $CD25^+$ T cells, which sometimes accompanies treatment with temozolomide,[23,27] may result in augmented immune status through suppression of the $CD4^+$ $CD25^+$ regulatory T cells. Sampson et al.[17] reported results of a Phase II multicenter clinical trial on the use of an EGFR variant III–specific peptide vaccine combined with temozolomide chemotherapy in patients with newly diagnosed EGFR variant III–positive glioblastoma, and according to their data, the median PFS of treated patients was 16 months and the median OS was not reached. Further investigation of such promising results is warranted.

Conclusions

The current study demonstrates that AFTV treatment concomitant with fractionated radiotherapy may be effective in patients with newly diagnosed glioblastoma and is not accompanied by severe toxicity. In light of these results, another clinical trial on the use of AFTV therapy during radiotherapy of newly diagnosed glioblastoma and concomitant chemotherapy with temozolomide is definitely needed.

Disclosure

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

Author contributions to the study and manuscript preparation include the following. Conception and design: Muragaki, Iseki, Takakura, Tsuboi, Matsumura, Matsutani, Sato, Ohno. Acquisition of data: Muragaki, Maruyama, Tanaka, Shinohara, Yamamoto. Analysis and interpretation of data: Muragaki, Iseki, Tsuboi, Yamamoto, Karasawa, Yamaguchi. Drafting the article: Muragaki, Shinohara. Critically revising the article: Maruyama, Iseki, Takakura, Yamamoto, Matsumura, Matsutani, Karasawa, Okada, Hori. Statistical analysis: Muragaki, Karasawa, Shimada, Yamaguchi. Administrative/technical/material support: Iseki, Shinohara, Takakura, Tsuboi, Matsumura, Matsutani, Karasawa, Ohno, Okada, Hori. Study supervision: Muragaki, Takakura, Hori. Editing of English text: Shinohara. Central review of histopathological specimens: Nakazato. Vaccine preparation: Sato, Uemae, Ohno.

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CLINICAL STUDY – PATIENT STUDY

A multicenter phase I trial of combination therapy with interferon-β and temozolomide for high-grade gliomas (INTEGRA study): the final report

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Abstract Our previous study demonstrated that interferon-β markedly enhanced chemosensitivity to temozolomide; one of the major mechanisms is downregulation of O^6-methylguanine DNA-methyltransferase transcription via p53 induction. This effect was also observed in an experimental animal model. The results of these studies suggest that compared to temozolomide-based chemotherapy performed concomitantly with radiotherapy, chemotherapy with interferon-β and temozolomide and concomitant radiotherapy might further improve the clinical outcomes of patients with malignant gliomas. A multicenter phase I clinical trial—the Integrated Japanese Multicenter Clinical Trial: a Phase I Study of Interferon-β and Temozolomide for Glioma in Combination with Radiotherapy (INTEGRA Study)—was conducted in patients with high-grade gliomas in order to evaluate the safety, feasibility, and preliminary clinical effectiveness of combination therapy with interferon-β and temozolomide. The primary endpoint was the incidence of adverse events. The exploratory endpoints were progression-free survival time and overall survival time. The study population comprised 16 patients with newly diagnosed and 7 patients with recurrent high-grade gliomas. Grades 3–4 leukocytopenia and neutropenia were observed in 6.7 and 13.3% of patients, respectively. Overall, 40% of patients showed an objective response to therapy. In patients with newly diagnosed glioblastoma, the median overall survival time was 17.1 months and the rate of 1-year progression-free survival was 50%. We conclude that this regimen is safe and well tolerated and may prolong survival of patients with glioblastoma. A phase II clinical study is essential to corroborate our findings.

Keywords Glioma · Interferon-β · Temozolomide

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Introduction

Gliomas account for approximately 40% of all brain tumors and are thus the most common primary tumors of the central nervous system (CNS). Primary brain tumors are classified according to cell type and histological grade into categories defined by the World Health Organization

(WHO) [1]. High-grade (WHO grades 3 and 4) gliomas, including anaplastic astrocytoma (AA), anaplastic oligodendroglioma (AO), anaplastic oligoastrocytoma (AOA), and glioblastoma multiforme (GBM), are often resistant to treatment [2–4]. Temozolomide (TMZ), an oral alkylating agent, has been shown to possess antitumor activity against malignant gliomas with minimal additional toxicity; furthermore, in a previous study, the median survival time substantially improved from 12 to 15 months when radiotherapy was concomitantly used with TMZ-based chemotherapy followed by adjuvant TMZ therapy [5]. In 2006, TMZ was approved by the National Ministry of Health and Welfare of Japan as the treatment agent for malignant gliomas, and a combination of radiotherapy and TMZ-based chemotherapy is now used as first-line therapy. However, the clinical outcome of TMZ therapy depends on the methylation status of the O^6-methylguanine-DNA methyltransferase (MGMT) promoter; patients with GBM whose tumors had the methylated MGMT promoter benefitted from TMZ compared to patients whose tumors had the unmethylated promoter [hazard ratio: 0.45; 95% confidence interval (CI): 0.32–0.61] [6]. Thus, MGMT modification is one of the key factors that could enhance the clinical benefits of this treatment.

Interferon (IFN)-β exerts pleiotropic biological effects and has been widely used either individually or in combination with other antitumor agents to treat malignant gliomas and melanomas [7]. In the treatment of malignant gliomas, IFN-β can act as a drug sensitizer, and it enhances the toxicity of chemotherapeutic agents against various neoplasms when it is administered in combination with nitrosourea. Combination therapy with IFN-β and nitrosourea has been used primarily for the treatment of gliomas in Japan [8]. In a previous in vitro study in human glioma cells, we found that IFN-β markedly enhanced chemosensitivity to TMZ [9]; this finding suggested that one of the major mechanisms by which IFN-β enhances chemosensitivity is the downregulation of MGMT transcription via p53 induction. This effect was also observed in an experimental animal model [10]. The results of these two studies suggested that compared to chemotherapy with TMZ alone and concomitant radiotherapy, chemotherapy with IFN-β, and TMZ with concomitant radiotherapy might further improve the clinical outcome of malignant gliomas. Here, in order to evaluate the safety, feasibility, and clinical effectiveness of combination therapy with IFN-β and TMZ, we conducted a phase I clinical study, the Integrated Japanese Multicenter Clinical Trial: a Phase I Study of Interferon-β and Temozolomide for Glioma in Combination with Radiotherapy (INTEGRA Study). This study involved eight medical institutions that covered the entire Japanese population.

Patients and methods

Patient population

We included patients fulfilling the following eligibility criteria: (1) newly diagnosed or recurrent high-grade gliomas (AA, AO, AOA, or GBM) as confirmed by histological analysis; (2) pretreatment magnetic resonance imaging (MRI) showing a tumor with >50% volume located in the supratentorial region except for the optic nerve, olfactory nerve, and pituitary gland; (3) age 18–75 years at the time of registration; (4) performance status (PS) of 0–2, or a PS of 3 only in the case of neurological deficit; (4) newly diagnosed high-grade gliomas for which chemoradiotherapy had not previously been performed; and (5) recurrent high-grade gliomas for which the time lapse since the end of prior antitumor therapy (e.g., chemotherapy, radiotherapy, and immunotherapy) was at least 4 weeks regardless of the regimen used. Additional inclusion criteria included adequate organ function before initiation of chemotherapy as defined on the basis of the following criteria: WBC count $\geq$3,000/mm^3 or neutrophil count $\geq$1,500/mm^3; platelet count $\geq$100,000/mm^3; hemoglobin level $\geq$8.0 g/dL; bilirubin level $\leq$1.5 mg/dL; serum glutamic oxaloacetic transaminase (SGOT) level $\leq$100 IU; serum glutamic pyruvic transaminase (SGPT) level $\leq$100 IU; creatinine level $\leq$1.5 mg/dL; creatinine clearance rate $\geq$50 ml/min; electrocardiogram (ECG) showing no serious arrhythmia; and absence of serious ischemic heart disease. All the patients were informed of the investigational nature of the study and were required to sign an informed consent form. The protocol was reviewed and approved by the institutional review boards of each participating institution. The following patients were excluded from the study: (1) those who had developed cancer synchronously or metachronously at 2 sites in the past 5 years; (2) those with confirmed carcinoma in situ; (3) those with meningitis or pneumonia; (4) women who were pregnant, possibly pregnant, or breastfeeding; (5) those with psychological disorders; (6) those with untreated diabetes mellitus (DM) or under insulin treatment for DM; (7) those who had a myocardial infarction in the past 3 months; and (8) those with a history of pulmonary fibrosis or interstitial pneumonia.

Study design and treatment

This study was a phase I, open-label, preliminary multicenter trial for evaluating the safety, feasibility, and clinical effectiveness of combination therapy with IFN-β for the treatment of malignant gliomas. The primary endpoint for the trial was the incidence of adverse events and the exploratory endpoints were progression-free survival time and overall survival time. In addition, the objective tumor

response was evaluated in a subpopulation of patients with measurable disease by the committees for safety and efficacy retrospectively. The reduction rate of measurable tumors was calculated according to the response evaluation criteria in solid tumors (RECIST) as assessed by MRI. Unmeasurable tumors were classified as those showing complete response (CR), partial response (PS), or progression (PD) or those that could not be evaluated (NE). Subsequently, overall response was evaluated on the basis of the results obtained for measurable and unmeasurable tumors. Pseudoprogression was excluded by carefully reviewing serial MRIs and case report forms including the information on steroid use and dose.

Patients with newly diagnosed high-grade gliomas received radiotherapy at a total dose of 60 Gy, intravenous (IV) IFN-β at a dose of 3 MIU/body on alternate days, and TMZ at a dose of 75 mg m^{-2} day^{-1}) daily. After the induction period, all the patients went through a 4-week washout period. Subsequently, the adjuvant treatment was initiated; this comprised IFN-β (3 MIU/body on the first morning of every 4th week) and TMZ (150 mg m^{-2} day^{-1}] on days 1–5 of the first cycle and 200 mg m^{-2} day^{-1}] on days 1–5 of the second to the sixth cycle). When no hematologic toxicity was noted, the TMZ dosage was increased to 200 mg m^{-2} day^{-1}) from the second cycle to the sixth cycle. The cycle was repeated 6 times every 28 days when no tumor progression or serious adverse events such as grade 4 hematologic toxicity were noted, and the patient did not refuse therapy or deviate from the protocol.

Patients with recurrent high-grade gliomas received a combination of IFN-β (3 MIU/body on the first morning of every 4th week) and TMZ (150 mg m^{-2} day^{-1} on days 1–5 of the first cycle and 200 mg m^{-2} day^{-1} on days 1–5 of the second to the sixth cycle). When no hematologic toxicity was noted, the TMZ dose was increased to 200 mg m^{-2} day^{-1} from the second cycle to the sixth cycle. All patients received non-steroidal anti-inflammatory drugs 1 h prior to IV IFN-β. On the basis of the results of previous clinical studies, this regimen is considered to be the most promising option [8, 11–14]. In this trial, we did not determine the maximum tolerated dose (MTD) of IFN-β for several reasons. IFN-β is a cytokine that exerts pleiotropic biological effects and has been widely used either individually or in combination with other antitumor agents for treating malignant gliomas and melanomas [7]. Combination therapy with IFN-β and nitrosourea (3 MIU/body in clinical setting) has been used primarily for the treatment of gliomas in Japan [8]. The favorable effect of a cytokine depends on its dose, and overdosing might not only increase the adverse events but also decrease the antitumor cytotoxic effect of the drug; therefore, it is difficult to determine the drug's optimal dose. However, on the basis of our previous animal study and our experience in the clinical use of IFN-β and nitrosourea, we concluded that the dosage of IFN-β used in this study would be the most promising and feasible one.

Registration and monitoring

The participating researchers were instructed to send an eligibility criteria report to the data center at Nagoya University, a third-party institution with which the study director was not affiliated. Patients were registered for 6 months starting December 2007. Laboratory data, including those from MRI, blood tests, and pathological tests, were obtained at the data center. The data quality was checked and verified at the data center. The committees for safety and efficacy (spearheaded by Dr. Kazuo Tabuchi, Koyanagi Memorial Hospital, Saga), radiotherapy (spearheaded by Dr. Shinji Naganawa, Department of Radiology, Nagoya University School of Medicine), pathological review (spearheaded by Dr. Youichi Nagasato, Department of Pathology, Gunma University School of Medicine), and statistics (spearheaded by Dr. Kunihiko Hayashi, Gunma University School of Health Science) submitted their reports to the head office.

Follow-up and statistical analysis

Disease progression and the occurrence of new tumors were examined by MRI performed at baseline and after at least 4–5 weeks of treatment. Blood tests were performed and symptoms were assessed before treatment and after at least 2 weeks during treatment. Follow-up continued for 3 months after the end of treatment. In cases in which therapy was discontinued because of toxicity, clinicians followed up the patients until they recovered. In addition, overall survival, progression-free survival, and treatment success curves were constructed as time-to-event plots with the Kaplan–Meier method.

Results

Patient characteristics

Between November 2006 and May 2007, 23 patients with high-grade gliomas were enrolled in our study. Detailed patient demographic and clinical characteristics are shown in Table 1. In order to evaluate the toxicity profile during maintenance treatment with the TMZ and IFN-β combination, we have included patients with recurrent high-grade gliomas for whom the time lapse since the end of prior antitumor therapy (e.g., chemotherapy, radiotherapy, and immunotherapy) was at least 4 weeks regardless of the regimen used.

Table 1 Demographic and clinical characteristics of patients

Characteristic	Value
Total	23
Age, years	
Median	51
Range	29–70
Sex, *n* (%)	
Male	10 (43%)
PS	
Median	1
Range	0–2
Histology	
Newly diagnosed	
GBM	10
AA	3
AO	2
AOA	1
Recurrent	7
GBM	3
AA	3
AO	1

PS performance status, *GBM* glioblastoma, *AA* anaplastic astrocytoma, *AO* anaplastic oligodendroglioma, *AOA* anaplastic oligoastrocytoma

Toxicity evaluation

Table 2 summarizes the nature of therapy-induced toxicity occurring during initial chemoradiotherapy. Grades 3–4 leukocytopenia and neutropenia were observed in 6.7 and 13.3% of patients, respectively. Grade 4 neutropenia recovered within 2 weeks without granulocyte colony-stimulating factor rescue. Hematologic toxicity was minimal during maintenance treatment. The most common adverse event was grade 1 appetite loss (30.4%) followed by grade 1 SGOT/SGPT elevation (26%). Grade 1 fever was observed in 15% of patients.

Response and survival

15 patients (10, newly diagnosed; 5, recurrent) with measurable disease were assessed for objective tumor response. Of these, 3 patients (20%) exhibited CR after 3 cycles of chemotherapy; 3 patients (20%) exhibited PR after 6 cycles; 5 patients (33%) exhibited stable disease after 6 cycles; and 4 patients (27%) exhibited disease progression after 1 cycle. Overall survival was assessed from the date of diagnosis to the date of the last follow-up or death. The overall survival distribution among patients with newly diagnosed high-grade gliomas (grade 3: 6 patients; grade 4: 10 patients) was estimated using the Kaplan–Meier method (Fig. 1). Progression-free survival was assessed from the date of diagnosis to the date of disease progression or death, whichever occurred first. The median survival time in patients with newly diagnosed grade 4 tumors was 17.1 months and the 1-year progression-free survival rate (95% CI) was 50.0% (range, 18.2–81.2%).

Table 2 Grade 3 and 4 toxicities attributed drug treatment

	Grade 3 (%)	Grade 4 (%)
Induction Tx		
Leucopenia	6.7	6.7
Platelet	0	0
Neutropenia	0	13.3
SGOT	0	0
SGPT	0	0
Maintenance Tx		
Leucopenia	5.6	0
Platelet	0	0
Neutropenia	0	0
SGOT	0	0
SGPT	0	0

Tx treatment

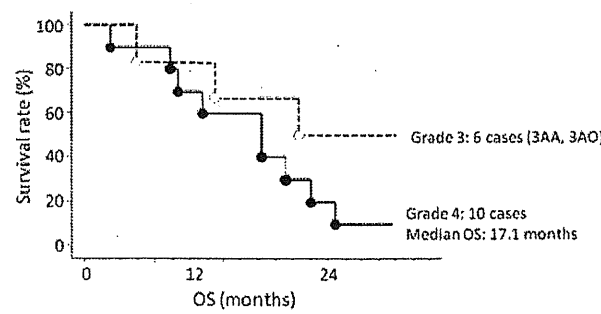

Fig. 1 The overall survival distribution among patients with newly diagnosed high-grade gliomas (grade 3: 6 patients; grade 4: 10 patients) was estimated using the Kaplan–Meier method. The median survival time in patients with newly diagnosed grade 4 tumors was 17.1 months. *AA* anaplastic astrocytome, *AO* anaplastic oligodendroglioma

Discussion

The results of this trial reveal that combination therapy with IFN-β and TMZ caused minimal toxicity. The most frequently observed toxic effect was the inhibition of hematopoiesis (e.g., with leukocytopenia); it took as long as 1 month after discontinuation of therapy for patients to recover from this effect. The overall response rate (CR+PR) was 40%, and the median survival time in newly diagnosed GBM patients was 17.1 months. Although the sample size was limited in this study, the median survival time was significantly longer than that in the EORTC 2698/22981 study

in which the median survival time in GBM patients under the Stupp regimen was 14.6 months.

In this trial, we did not assess the MGMT status of patients because it was not a part of the protocol [15]. However, we retrospectively reviewed the cases of 68 consecutive patients with newly diagnosed GBM. Of these patients, 57.4% received a combination of IFN-β and TMZ. When this combination was administered at a similar dosage in our phase I trial, a median survival time of 19.9 months was achieved, whereas the median survival time achieved with TMZ therapy alone was 12.7 months. Notably, in patients whose tumors had the unmethylated MGMT promoter, the median survival time increased to 17.2 months after TMZ with IFN-β therapy compared to 12.5 months after TMZ without IFN-β therapy. This finding suggests that combination IFN-β and TMZ therapy may improve the clinical outcomes in patients with tumors expressing MGMT with an unmethylated MGMT promoter [16]. This finding indirectly supports the hypothesis that downregulation of MGMT expression might have contributed to the clinical efficacy of the combination used in this study.

The phase I trial of combination therapy with IFN-β and TMZ reported in this paper has defined the therapeutic approach for our ongoing phase II trial for the same; patients with newly diagnosed GBM will be included in the phase II trial. This trial will provide the data required to determine whether IFN-β inclusion will enhance the clinical efficacy of TMZ-based chemotherapy performed concomitantly with radiotherapy.

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Original Article

Benefits of Interferon-β and Temozolomide Combination Therapy for Newly Diagnosed Primary Glioblastoma With the Unmethylated MGMT Promoter

A Multicenter Study

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BACKGROUND: The aim of the current study was to catalog genomic and epigenomic abnormalities in newly diagnosed glioblastoma patients and determine the correlation among clinical, genetic, and epigenetic profiles and clinical outcome. **METHODS:** This study retrospectively included 68 consecutive patients who underwent surgical treatment and received standard radiotherapy with temozolomide (TMZ)-based chemotherapy. Of a total of 68 patients, 39 patients (57.4%) received interferon (IFN)-β in combination of TMZ. **RESULTS:** The genetic and epigenetic alterations frequently observed were *EGFR* amplification (51.5%), *TP53* mutation (33.8%), *CDKN2A* loss (32.4%), *TP53* loss (16.2%), methylation of the MGMT promoter (33.8%) and *IDH1* mutation (5.9%). Multivariate analysis revealed that methylated MGMT promoter and the combination of TMZ and IFN-β were independent prognostic factors associated with survival. The median survival time (MST) of the patients who received the combination of IFN-β and TMZ was significantly greater with 19.9 months as compared to the TMZ alone group (12.7 months). Notably, in even patients whose tumors had unmethylated MGMT promoter, the MST prolonged to 17.2 months when receiving TMZ with IFN-β, compared to 12.5 months in those receiving TMZ without IFN-β. **CONCLUSIONS:** Taken together, addition of IFN-β for newly diagnosed primary GBM achieved a favorable outcome, particularly in patients with unmethylated MGMT promoter. *Cancer* 2010;000:000–000. © *2010 American Cancer Society.*

KEYWORDS: IDH1, MGMT methylation, glioblastoma, interferon-β, temozolomide.

Glioblastoma multiforme (GBM) is one of the most frequent primary brain tumors in the central nervous system in adults and is highly malignant, with a median survival time of about one year from diagnosis. This is despite aggressive treatment, surgery, postoperative radiotherapy, and adjuvant chemotherapy. An international randomized trial by the European Organization for Research and Treatment of Cancer/National Cancer Institute of Canada (EORTC/NCIC) comparing radiotherapy alone and concomitant radiotherapy and temozolomide (TMZ) clearly attested the benefits of adjuvant TMZ chemotherapy for GBM patients.[1] Since then, TMZ has been the current first-line chemotherapeutic agent for GBM.

A subanalysis in this trial showed the effectiveness of epigenetic silencing of the MGMT gene by promoter methylation with longer survival in patients with primary GBM; it also suggested the benefits of combining chemotherapy using TMZ with radiotherapy.[2]

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Furthermore, there have been recent attempts to comprehensively profile GBM genes by The Cancer Genome Atlas (TCGA) project and other groups.[3,4] Some genetic aberrations in GBM, such as *TP53* mutation or deletion, *NF1* deletion or mutation, and *ERBB2* mutation, have been found to be more common than previously reported. In addition, novel molecular markers, such as frequent mutations of the *IDH1* and *IDH2* genes in secondary GBM have been discovered.[5-7] These findings on mutations, genomic and epigenomic aberrations, and transcriptomal features in GBM might aid in understanding the classification of GBM and its further potential clinical implications.

However, the TCGA project included GBM patients who received surgical treatment, and detailed information on adjuvant chemoradiotherapy was not provided. Therefore, the close relationship between the gene profile provided by TCGA and chemotherapy regimens remains unknown.[3]

In this current study, we aimed to determine the correlation between clinical, genetic, and epigenetic profiles, and clinical outcome in newly diagnosed GBM patients who received TMZ-based chemotherapy. Interestingly, we found a significant beneficial outcome in patients receiving TMZ in addition to IFN-β. Moreover, our study discovered that GBM patients with the ummethylated O^6-methylguanine-DNA methyltransferase (MGMT) promoter, in particular, showed benefits from IFN-β.

MATERIALS AND METHODS

Patient population

We retrospectively reviewed 68 consecutive patients with newly diagnosed primary GBM who underwent surgical treatment at several academic tertiary-care neurosurgical institutions: Nagoya University Hospital, Hamamatsu University Hospital, Oita University Hospital, and Shizuoka Cancer Center from May 2006 through June 2010 after TMZ was approved as the treatment agent for malignant gliomas by the National Ministry of Health and Welfare of Japan. The diagnosis of GBM was established by histological confirmation according to the WHO guidelines[8,9] independently by at least two expert neuropathologists. The clinical, operative, and hospital course records were reviewed. Information collected from clinical notes included patient demographics, pre- and postoperative neuroimaging, and adjuvant therapy. Preoperative Eastern Cooperative Oncology Group performance status (ECOG PS) scores were assigned by the clinician at the time of evaluation and were available in the chart for review for all patients. The study was approved by the institutional review board at each participating hospital and complied with all provisions of the Declaration of Helsinki.

Treatment

Radiotherapy

After undergoing surgery, the patients received focal external-beam radiotherapy by conventional radiation planning to approximately 60 Gray (Gy) (±5% total dose), with daily concurrent TMZ at 75 mg/m^2 throughout the course of radiotherapy.

Chemotherapy

All patients received the standard Stupp regimen.[1] In the absence of grade 3 or 4 hematological excessive toxicity, TMZ administration was continued until clinical or radiological evidence of disease progression was observed. Of these 68 patients, 39 patients (57.4%) received adjuvant IFN-β treatment (Table 1). Patients in Nagoya University and Oita University received chemotherapy consisting of IFN-β. There were no significant differences in any of the clinical parameters and genetic, epigenetic parameters (i.e., age, sex, preoperative PS, tumor location, extent of resection, genetic and epigenetic alterations between the institutions using regimen with and without IFN-β. The IFN-β chemotherapy regimen comprised 3 million international units (MIU)/body administered intravenously on alternate days during radiotherapy and TMZ-induction chemotherapy.[10,11] At the end of the induction period, after a 4-week interval, the patients were administered 3 MIU/body of IFN-β on the first morning every 4 weeks during TMZ maintenance chemotherapy. In the case of tumor progression, salvage or second-line therapy was administered at the investigators' discretion; most patients received additional chemotherapy.

Response Evaluation During Treatment

Both radiological and clinical findings were used to evaluate the response. Follow-up magnetic resonance imaging (MRI) was performed for alternate cycles. If the MRI showed continued increase in enhancement, the case was considered as tumor progression. If re-resection was performed for a recurrent mass lesion, histological interpretation formed the basis for definitive diagnosis (treatment-related necrosis vs recurrent tumor).

Table 1. Clinical Characteristics[a]{TC}

Parameter	No. of Patients	%
	n=68	
Age(y)		
Median	55.0	
Range	12-84	
<40	12	17.6
≥40, <60	24	35.3
≥60	32	47.1
Sex		
Male	41	60.3
Female	27	39.7
Preoperative ECOG performance status		
Median	1	
Range	0-3	
Preoperative ECOG performance status		
≤1	45	66.2
>1	23	33.8
Tumor location		
Superficial	50	73.5
Deep	18	26.5
Surgery		
GTR	24	35.3
Non-GTR	44	64.7
Chemotherapy		
TMZ only	29	42.6
TMZ+ IFN-β	39	57.4

ECOG indicates Eastern Cooperative Oncology Group; PS, performance status; GTR, macroscopic (gross) total removal; TMZ, temozolomide.

Tumor samples and DNA Extraction

All patients provided their written informed consent for molecular studies of their tumor, and the protocol was approved by the ethics committee at each center. Sixty-eight brain tumor specimens were obtained at the time of first surgical resection.

Tumor tissue samples were immediately frozen and stored at −80°C until the extraction of genomic DNA. DNA was prepared using the QIAmp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Placental DNA was used as the normal control. The amount of DNA obtained from the tumor was sufficient for the subsequent genomic and epigenomic analyses.

Multiplex Ligation-Dependent Probe Amplification

Multiplex ligation-dependent probe amplification (MLPA) was used for the determination of allelic losses and gains of the gene in the tumor samples. The analysis was performed using the SALSA MLPA KIT P088-B1 and P105-C1 in accordance with the manufacturer's protocol (MRC Holland, Amsterdam, Netherland).[12-15] Information regarding the probe sequences and ligation sites can be found at www.mlpa.com. Amplification products were separated on an ABI® 3130 × I Genetic Analyzer (Applied Biosystems, Foster City, CA) and quantified with Genemapper 4.0 software (Applied Biosystems). Duplicate experiments were performed to obtain accurate MLPA values. Data analysis was performed with an original Excel-based program based on MRC-Holland's procedures. Normalization for sample data was first performed on control probes, and each tumor sample was then normalized using the data on 2 control samples, using peripheral blood DNA. Single regression for control and tumor data slope correction was performed. Abnormal/normal ratio limits were set at 0.65 and 1.3. Statistical analysis was performed using the same Coffalyser software.

Pyrosequencing

Tumor DNA was modified with bisulfate using the EpiTect bisulfite kit (Qiagen, Courtaboeuf Cedex, France). Pyrosequencing technology was used to determine the methylation status of the CpG island region of MGMT as described previously.[16,17] We used the touchdown PCR method. The primer sequences used were the MGMT forward primer, 5′-TTGGTAAATTAAGGTATAGAGTTTT-3′, and the MGMT biotinylated reverse primer, 5′-AAA CAATCTACGCATCCT-3′. PCR included a denaturation step at 95°C for 30 s, followed by annealing at various temperatures for 45 s, and extension at 72°C for 45 s. After PCR, the biotinylated PCR product was purified as recommended by the manufacturer. In brief, the PCR product was bound to Streptavidin Sepharose HP (Amersham Biosciences, Uppsala, Sweden), and the Sepharose beads containing the immobilized PCR product were purified, washed, and denatured using 0.2 N NaOH solution and washed again. Next, 0.3 mM pyrosequencing primer was annealed to the purified single-stranded PCR product, and pyrosequencing was performed using the PSQ HS 96 Pyrosequencing System (Pyrosequencing, Westborough, MA). The pyrosequencing primer was 5′-GGAAGTTGGGAAGG-3′. Methylation quantification was performed using the provided software.

TP53 and IDH1/IDH2 Sequencing

Direct sequencing of the *TP53* exons 5 to 8 and *IDH1/IDH2* was performed as previously described.[7,18,19] The primer sequences are listed in Table 2.

Table 2. List of Primer Sequences for Direct DNA Sequencing{TC}

Gene name	Exon		Sequence
TP53	Exon 5	F	5′-TTATCTGTTCACTTGTGCCC-3′
		R	5′-ACCCTGGGCAACCAGCCCTG-3′
	Exon 6	F	5′-ACGACAGGGCTGGTTGCCCA-3′,
		R	5′-CTCCCAGAGACCCCAGTTGC-3′
	Exon 7	F	5′-GGCCTCATCTTGGGCCTGTG-3′
		R	5′-CAGTGTGCAGGGTGGCAAGT-3′
	Exon 8	F	5′-CTGCCTCTTGCTTCTCTTTT-3′
		R	5′-TCTCCTCCACCGCTTCTTGT-3′.
IDH1		F	5′-CGGTCTTCAGAGAAGCCATT-3′
		R	5′-GCAAAATCACATTATTGCCAAC-3′
IDH2		F	5′-AGCCCATCATCTGCAAAAAC-3′
		R	5′-CTAGGCGAGGAGCTCCAGT-3′.

F indicates forward primer; R, reverse primer;.

For IDH sequencing, a fragment 129 bp in length, spanning the sequence encoding the catalytic domain of *IDH1*, including codon 132, and a fragment 150 bp in length spanning the sequence encoding the catalytic domain of *IDH2*, including codon 172, were amplified. We applied touchdown PCR, using the standard buffer conditions: it comprised 5 ng of DNA and AmpliTaq Gold DNA Polymerase (Applied Biosystems) run for 16 cycles with denaturation at 95°C for 30 s, annealing at 65 to 57°C (decreasing by 0.5°C per cycle) for 30 s, and extension at 72°C for 60 s in a total volume of 12.5 μl and add 30 cycles with denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s, ending with at 72°C for 7 min to complete extension.

Direct sequencing was performed using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). The reactions were carried out using an ABI 3100 Genetic Analyzer (Applied Biosystems).

Statistical analysis

Statistical analysis was performed using the statistical software SPSS for Windows, version 17.0 (SPSS Inc, Chicago, Ill). The Mann-Whitney U test, χ^2 test, and Fisher exact test were used to test for association of clinical variables and molecular markers. Survival was estimated by using the Kaplan-Meier method, and survival curves were compared by using the log-rank test. Progression-free survival (PFS) was calculated from the day of first surgery until tumor progression, death, or end of follow up. Overall survival (OS) was calculated from the day of first surgery until death or the end of follow up. Univariate and multivariate analyses were performed to test the potential influence of baseline characteristics on survival. The effect of each single molecular marker on PFS and OS was investigated using the Cox proportional hazards model, adjusting for the major clinical prognostic factors, including age at diagnosis (<40 vs ≥40, <60 vs ≥60 years), ECOG performance status score (ECOG PS; ≤1 vs >1), extent of resection (macroscopic [gross] total resection [GTR] vs non-GTR), tumor location (superficial vs deep), MGMT promoter methylation status, chromosome 1p loss of heterozygosity (LOH), 19qLOH, *PTEN* loss, *CDKN2A* loss, *TP53* loss and mutation, *ERBB2* amplification, *EGFR* amplification, *IDH1* and *IDH2* mutation, and adjuvant therapy (with IFN-β vs without IFN-β). Factors with no significant association with survival, at a level of more than 0.05 in the multivariate analysis, were eliminated. The remaining factors in the multivariate proportional hazard model (P <.05) were considered to be independent predictors of survival.

To assess for the treatment effects of TMZ with IFN-β versus TMZ without IFN-β for overall survival (OS), the hazard ratio was computed using a proportional hazard model by baseline characteristics in stratified analysis.

RESULTS

Clinical parameters

Between May 2006 and June 2010, 68 consecutive patients newly diagnosed with primary GBM were registered in this study. Their clinical characteristics are summarized in Table 1. This study group comprised 41 men and 27 women aged 12-84 years (median, 55). The median preoperative ECOG PS score at diagnosis was 1 (range, 0-3); the preoperative ECOG PS score was <1 in 45 patients (66.2%). All tumors were located in the supratentorial region: 50 tumors were located in the superficial area (cortical or subcortical area), and 18 were located in deep anatomical structures such as the basal ganglia and corpus callosum. No tumor was noted in the optic nerve, olfactory nerve, and pituitary gland on pretreatment MRI. No tumor dissemination was detected by MRI. Surgical GTR was achieved in 24 patients (35.3%), and 44 patients underwent non-GTR (64.7%). None of the patients had concurrent active malignancy, and the baseline organ function before chemotherapy was as follows: absolute WBC ≥3000/mm^3 or neutrophil count ≥1,500/mm^3, platelet count ≥100,000/mm^3, hemoglobin ≥8.0 g/dl, AST less than 2.5 × the upper limit of normal (ULN), total bilirubin 2 × ULN, and creatinine 2 × ULN, and electrocardiogram showing no serious