

Selection of chemotherapy for glioblastoma expressing *O*⁶-methylguanine-DNA methyltransferase

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Abstract. The therapeutic benefit of nitrosoureas or temozolomide for glioblastoma is limited mainly by *O*⁶-methylguanine-DNA methyltransferase (MGMT) expression. The aim of this study was to evaluate the effectiveness of various anticancer drugs for MGMT-positive glioblastoma. Seventy-four glioblastoma patients were administered various anticancer drugs according to drug sensitivity testing. For the individualization, drug-induced apoptosis was quantified by flow cytometry in the primary culture of surgically resected tumor cells. The MGMT protein expression was analyzed by immunohistochemistry. The median survival of the patients receiving the individualized chemotherapy was 19.4 months (95% CI, 15.9-22.1). The patients with negative MGMT immunostaining had significantly longer survival than those with positive MGMT immunostaining [median survival, 22.3 months (95% CI, 17.6-27.0) vs. 15.1 months (95% CI, 13.4-16.8); *p*=0.0188]. For MGMT-positive tumors, the platinum agents and the taxanes were more frequently selected for administration than the other categories of anticancer agents. The patient survival period of MGMT-positive glioblastomas treated with the platinum agents or the taxanes [median survival, 20.1 months (95% CI, 18.0-22.7)] was significantly longer than that of MGMT-positive tumors treated with nitrosoureas (*p*=0.0026), and was equivalent to that of MGMT-negative glioblastomas (*p*=0.3047). These results suggest that the platinum agents and the taxanes offer the best probability to be effective against immunohistochemically MGMT-positive glioblastomas.

Introduction

The currently available optimum treatment for glioblastoma consists of cytoreductive surgery followed by radiotherapy

and chemotherapy (1). This conventional therapeutic strategy results in a median survival of 12-15 months in consecutive, non-selected glioblastoma patients (2-4). Most large-scale clinical studies on chemotherapy for malignant gliomas have utilized nitrosoureas (2,3), and have usually produced negative results regarding the survival gain for glioblastoma patients. Although temozolomide chemotherapy contributes to a significant improvement in patient survival, its benefit is usually restricted to tumors without *O*⁶-methylguanine-DNA methyltransferase (MGMT) expression (4,5). Since the level of MGMT expression strongly influences the efficacy of nitrosoureas or temozolomide (5-9), the establishment of novel therapeutic strategies for MGMT-positive glioblastoma is one of the main issues in contemporary neurooncology.

Current cancer treatments for categories of patients generally require the selection of therapy made on the basis of clinical trials conducted on large populations. However, the heterogeneity in drug sensitivity partly reduces the clinical success gained with these empiric chemotherapeutic regimens used for the general patient population (1). A therapeutic strategy with a protocol modified case by case according to drug sensitivity is termed 'individualized' or 'tailor-made' chemotherapy (10). Published clinical studies using *in vitro* drug sensitivity tests (DST) have shown improved patient response rates as compared with empiric regimens (11-15). The lessons learned from individualized chemotherapy may be valuable in planning chemotherapy regimens for glioblastoma as various anticancer drugs are actually administered in clinics.

We treated glioblastoma patients with various anticancer agents according to individualized protocols selected by DST. However, individualization of chemotherapy cannot easily be adopted in every institution, since it is both time-consuming and non-economical. In this report, the efficacy of each anticancer agent for glioblastoma was retrospectively examined in relation to the MGMT expression status by immunohistochemistry. This information provides a clue for the selection of anticancer drugs against glioblastoma expressing a high level of MGMT or those harboring unmethylated promoter of the MGMT gene.

Materials and methods

Patients. Seventy-four consecutive patients newly diagnosed with glioblastoma according to WHO classification were

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treated with individualized chemotherapy at Chiba University Hospital or Chiba Cancer Center Hospital from 1995 to 2004. All of the patients treated during this period were evaluated and included in the study without exclusion. The study protocol was approved by the institutional review board, and a written informed consent was obtained from all of the patients or a guardian. The patient characteristics are summarized in Table I. Magnetic resonance imaging (MRI), with and without gadolinium enhancement, was performed preoperatively and postoperatively before the initiation of radio-chemotherapy. Regarding extent of resection, the total/subtotal resection was defined as 90% or more reduction of the tumor volume in the postoperative MRI. The biopsy meant the CT-guided stereotactic needle biopsy, and partial removal covered all other situations. Toxicity was graded according to the National Cancer Institute's Common Toxicity Criteria version 3.0.

Drug sensitivity test (DST). Direct quantification of apoptosis by means of flow cytometric DNA analysis is widely used in basic research and has been successfully utilized for clinical DST (15-17). Cell suspensions prepared from surgically resected tumor tissues were incubated with each of 25 different anticancer drugs already being used in clinical practice (cyclophosphamide, ifosfamide, nimustine, ranimustine, cisplatin, carboplatin, adriamycin, daunomycin, pirarubicin, epirubicin, aclarubicin, mitoxantrone, etoposide, camptothecin, methotrexate, 5-fluorouracil, thioinosine, cytosine arabinoside, mitomycin C, bleomycin, vincristine, vinblastine, vindesine, paclitaxel and docetaxel). The *in vitro* drug concentrations were set both at the peak plasma concentration when the clinically recommended doses were provided and at 1/10 of that level (18). Drug-induced apoptosis was quantified with a flow cytometer (FACScan; Becton Dickinson, Mountain View, CA, USA) as the sub-G1 population. To confirm the presence of drug-induced apoptosis, morphological examinations of the nuclei were also performed on the same samples. DNA integrity assessed by the FCM analysis correlated well with the morphological changes in the nuclei.

Treatment protocols. For individualization of chemotherapy, the most effective drug *in vitro* was routinely selected as the key drug for each individual patient. In addition, one or two drugs were selected for combination with the key drug according to their degree of effectiveness and their mechanism of pharmaceutical action. The doses and schedules of chemotherapy regimens were determined on the basis of clinically recommended doses. When no agent was positive *in vitro*, the patients were treated with a modified PCV chemotherapy with substitution of lomustine with nimustine (nimustine 75 mg/m², vincristine 1 mg/m² and procarbazine 100 mg/day) (19). For all patients, the conventional 60-Gy radiotherapy with a megavoltage machine was started within 2 weeks of surgical removal in conjunction with the chemotherapy.

MGMT immunohistochemistry. For immunohistochemical analysis, paraffin-embedded samples were sliced and mounted on glass slides. Mouse monoclonal anti-MGMT antibody MT3.1 (1:200 dilution; Chemicon, Inc., Temecula, CA, USA) was used as the primary antibody. A heat-induced epitope was formed using microwaves in 10 mM citric acid buffer

Table I. Patient characteristics (n=74).

Age (years)	
Mean	51.5
Range	15-77
Gender	
Male	49 (66%)
Female	25 (34%)
Karnofsky performance score	
≥70	42 (57%)
<70	32 (43%)
Tumor location	
Left	37 (50%)
Right	28 (38%)
Midline	9 (12%)
Extent of surgery	
Total/Subtotal	46 (62%)
Partial/Biopsy	28 (38%)

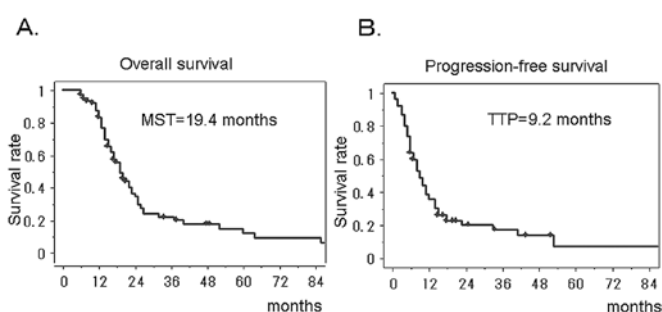


Figure 1. Kaplan-Meier analyses of overall survival (A) and progression-free survival (B) in the patients with glioblastoma treated with individualized chemotherapy. MST, median survival time; TTP, time to tumor progression.

at pH 7.2. The samples were incubated with the antibody overnight in the same buffer followed by incubation with the biotinylated secondary antibody (1:500 dilution; Dako, Tokyo, Japan). The bound antibodies were visualized by the avidin biotinylated peroxidase complex method and diaminobenzidine tetrachloride (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Human liver was used as the positive control, and the negative control was achieved by omitting the primary antibody from the procedure. Tissue specimens that showed staining of >10% of the malignant cells were considered positive for MGMT.

Statistical analysis. The primary end-point of this study was overall survival and the secondary end-points were progression-free survival and safety. Survival curves were generated using the Kaplan-Meier method, and the survival rates were compared with the log-rank test. The patient survival duration was calculated from the date of surgery until the date of last follow-up or death, and progression-free survival until the date of recurrence detection or until the last follow-up. The Fisher's exact probability test and χ^2 test were used to evaluate

Table II. Multivariate analyses for favorable prognostic factors.

	Hazard ratio (95% CI)	P-value
Age (<50 vs. ≥50)	0.41 (0.26-0.66)	0.0002
Karnofsky performance score (≥70 vs. <70)	0.69 (0.47-1.00)	0.0607
MGMT expression (negative vs. positive)	0.59 (0.41-0.88)	0.0081
Extent of resection (≥90 vs. <90)	0.60 (0.39-0.93)	0.0211

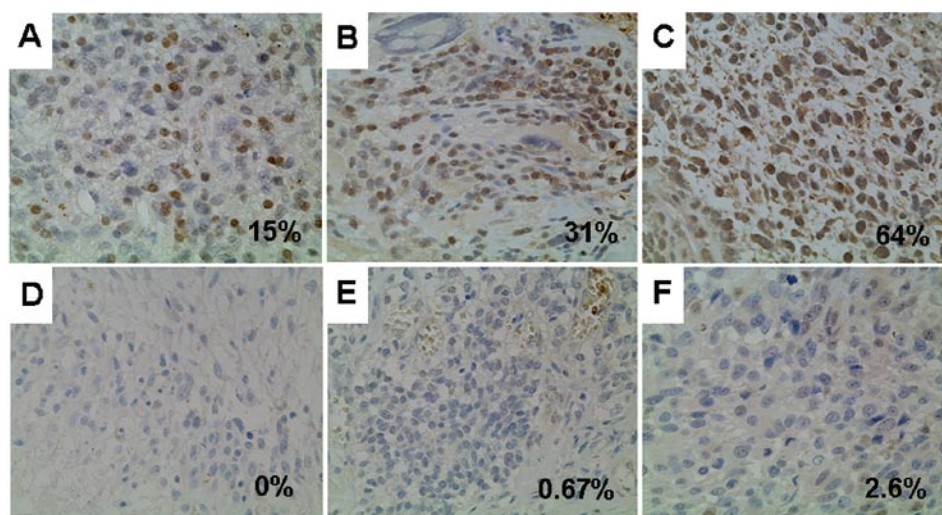


Figure 2. Immunohistochemical analyses for MGMT expression. The representative cases for positive (A, B and C) and negative (D, E and F) immunostaining. The cut-off point for positive MGMT expression was set at 10%.

the statistical significance of the differences between patient characteristics of the two groups. Cox's proportional hazard model was used to analyze the prognostic variables. The hazard ratios for death were calculated considering adjustment for age, Karnofsky performance status score and the extent of resection.

Results

Efficacy of individualized chemotherapy. All specimens from the 74 patients were examined for their *in vitro* susceptibility to the 25 anticancer drugs. In this series of newly diagnosed glioblastoma patients, the success rate of the DST was 100%. There was remarkable heterogeneity in the most effective drug. The median survival time of all of the 74 glioblastoma patients treated with the individualized chemotherapy was 19.4 months (95% CI, 15.9-22.1), and the 2-year survival rate was 36.5% (95% CI, 24.3-48.7). The median progression-free survival was 9.2 months (95% CI, 7.6-12.3) (Fig. 1). The survival periods could be favorably compared with those treated with temozolomide, the present-day standard regimen for glioblastoma.

The univariate analysis showed that the clinical factors previously known to affect the survival of patients with glioblastoma were correlated with favorable prognosis in this study; a Karnofsky performance status score of ≥70%, tumor resection of ≥90% and <50 years of age. The multivariate analysis showed that <50 years of age and tumor resection of

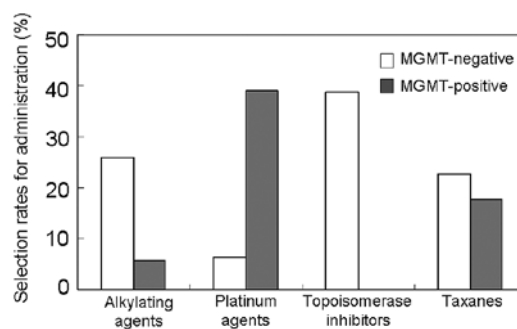


Figure 3. Selection rates of each anticancer drug as a key drug for administration; comparisons between the MGMT-negative and -positive glioblastomas.

≥90% were significantly associated with favorable prognosis (Table II, $p=0.0002$ and $p=0.0211$, respectively).

MGMT expression and chemosensitivity. The MGMT-positive rate was 53.7% for the 74 glioblastomas (Fig. 2). According to the DST, 58 tumors (78%) had at least one effective drug, and the other 16 tumors (22%) were negative for all of the 25 anticancer drugs examined (all-drug-resistant tumors). The relationship between the MGMT expression status and chemosensitivity was analyzed (Fig. 3). For the MGMT-positive tumors, the alkylating agents were selected only in two cases, and the topoisomerase inhibitors were never selected for administration as a key drug. The platinum agents were

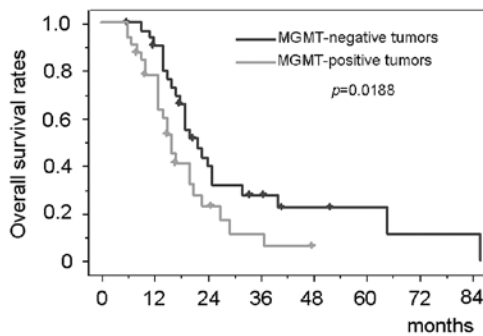


Figure 4. Kaplan-Meier analyses of the overall survival compared between MGMT-negative and MGMT-positive glioblastoma patients treated with individualized chemotherapy.

more frequently effective against the MGMT-positive tumors than against the MGMT-negative tumors. The taxanes were equally selected either in the MGMT-negative or the MGMT-positive group. Most of the all-drug-resistant tumors (14 out of 16 cases, 87.5%) were included in the MGMT-positive group ($p=0.0019$). Thus, the MGMT expression status significantly influenced the *in vitro* chemosensitivity of almost all categories of anticancer agents except for the taxanes.

MGMT expression and survival. The patients with negative MGMT immunostaining had significantly longer survival than those with positive MGMT [median survival, 22.3 months (95% CI, 17.6-27.0) vs. 15.1 months (95% CI, 13.4-16.8); $p=0.0188$] (Fig. 4). Immunohistochemical MGMT expression status had a significant impact on the survival period in the multivariate analysis (Table II). The survival period of the patients with MGMT-positive tumors treated with the platinum agents or the taxanes [median survival, 20.1 months (95% CI, 18.0-22.7)] was equivalent to that of the MGMT-negative tumors ($p=0.3047$) (Fig. 5). In contrast, the survival time of the patients with all-drug-resistant MGMT-positive tumors ($n=14$) who were treated with the nitrosourea-based chemotherapy (the modified PCV therapy) [median survival, 13.0 months (95% CI, 11.4-14.6)] was significantly shorter than both that of the patients with MGMT-negative tumors ($p=0.0007$) and that of MGMT-positive tumors treated with the platinum agents or the taxanes ($p=0.0026$).

Safety evaluation. We monitored the adverse events, with special focus on the hematological toxic effects. They were graded according to the National Cancer Institute's Common Toxicity Criteria version 3.0. Grade 3 or 4 neutropenia was observed in 9 patients (12.2%), and severe pneumonia occurred in 3 patients (4.1%). However, there was no treatment-related death in the present series.

Discussion

Our results suggest that individualized chemotherapy with anticancer drugs prospectively selected based on *in vitro* chemosensitivity tests for each glioblastoma patient provides a median survival of 19.4 months which compares favorably with most of the previously reported studies (2-4). The potentially poor prognosis groups with age greater than

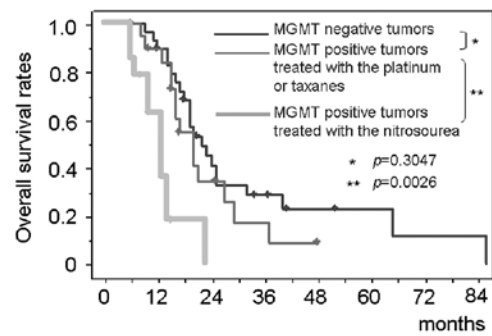


Figure 5. Kaplan-Meier analyses of the overall patient survival compared among MGMT-negative glioblastoma, MGMT-positive tumors treated with nitrosourea and those treated with the platinum agents or the taxanes.

50 years, Karnofsky performance status score less than 70% and surgical resection less than 90% particularly benefited from the individualized chemotherapy. Therefore, this study suggests an important new direction in the chemotherapy for glioblastoma.

An important implication of this study is that a large number of MGMT-positive glioblastomas were effectively treated with individualized chemotherapy. Many studies have indicated that MGMT is a significant prognostic factor for shorter survival rates in glioblastoma patients (5-7), whereas its prognostic value remains controversial (8,9). Efficacy of temozolomide also depends significantly on the level of MGMT expression (5). Therefore, one of the most important issues in contemporary neurooncology is how to treat glioblastoma with high MGMT expression. Therapeutic strategies for MGMT-positive glioblastoma currently under consideration have been designed to deplete MGMT and to combine other agents which are not affected by MGMT (21). The present results demonstrate that currently available anticancer drugs are much more effective when administered to those most likely to respond. An individualized or tailored strategy based on multiple biological information of the tumor would be one of the effective approaches to treat MGMT-positive glioblastoma.

However, an individualization strategy cannot easily be adopted in every institution. The lessons learned from individualized chemotherapy may be valuable in planning chemotherapy regimens for MGMT-positive glioblastoma. The present study suggests that the platinum agents and the taxanes can potentially prolong the survival of patients with MGMT-positive glioblastoma. The platinum agents as well as temozolomide and *O*⁶-benzylguanine can abrogate MGMT activity (22). Several studies have also shown that the antitumor activity of platinum agents is not affected by MGMT activity (23-25). This knowledge has led to Phase II clinical trials with promising results (26-28). Although MGMT affected the efficacies of diverse anticancer drugs, only the taxanes were independent from the MGMT status. Taxanes were clinically used in some trials without marked improvement in the efficacy for glioblastoma (29-31). However, multiple molecular mechanisms were reported to affect their efficacies for glioma cells (32). It is preferable to treat MGMT-positive glioblastoma with multi-modality regimens including platinum agents or the taxanes.

Currently, MGMT expression is estimated mainly by methylation-specific PCR or immunohistochemistry (5-9). Methylation-specific PCR is highly sensitive but is unable to assess intratumoral heterogeneity and contaminating normal cells such as endothelial cells (8,9). A methylated band is observed even when cells that carry MGMT promoter hypermethylation represent only a minor portion of the tumor. Regulation of MGMT expression is a more complex phenomenon in which abnormal promoter methylation is not the sole determining factor. We employed immunohistochemistry to directly evaluate the final functional molecule and the intratumoral heterogeneity, although an objective threshold for evaluation may not be easily set. We used a cut-off value of 10%, whereas the reported values vary from 5 to 35% (8,9,33,34). The results showed a tendency of polarization of the MGMT-positive rate in the tumors with rates of less than 5% and those with more than 35%. Consequently, the overall positive rate in our study is consistent with published reports employing immunohistochemistry.

This is the first report to show that individualization of chemotherapy can potentially prolong the survival of non-selected consecutive glioblastoma patients with high MGMT expression without any additional toxicity. When the stratification based on the MGMT expression is available, platinum agents or the taxanes offer the highest probability for effectiveness against MGMT-positive glioblastomas.

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