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Individual adjuvant therapy for malignant gliomas based on O^6-methylguanine-DNA methyltransferase messenger RNA quantitation by real-time reverse-transcription polymerase chain-reaction

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Abstract. A new adjuvant therapy, individual adjuvant therapy (IAT), which is individualized according to the results of real-time reverse-transcription polymerase chain-reaction (RT-PCR) for O^6-methylguanine-DNA methyltransferase (MGMT), was used to treat malignant gliomas. Immediately after the operation, mRNA expression for drug-resistance genes was investigated in frozen samples of malignant gliomas from 55 patients (30 glioblastoma multiformes, 20 anaplastic astrocytomas and 5 anaplastic oligodendroglial tumors) by real-time quantitative RT-PCR with specific primers for MGMT. Forty-two patients were treated with 1-(4-amino-2-methyl-5-pyrimidynyl) methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride (ACNU)-based chemotherapies since the relative quantitation value (RQV) of MGMT in real-time RT-PCR with SYBR-Green I was <1.0 or the absolute value of MGMT mRNA as measured by Taq Man probe methods normalized to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was $<6.0\times10^3$ copies/μg RNA. Thirteen patients, whose tumors had an RQV of >1.0 or who had an absolute value of MGMT of $>6.0\times10^3$ copies/μg RNA, were treated by platinum-based chemotherapy using cisplatin or carboplatin. The response rate was 40.9% for glioblastoma multiformes, 60.0% for anaplastic astrocytomas and 80.0% for anaplastic oligodendroglial tumors. The median survival period of 30 patients with glioblastoma treated by IAT was 21.7 months. The 2-year survival rate of glioblastoma patients treated by IAT was 70.9%. Our IAT, based on the results of real-time RT-PCR, may lead to a beneficial glioma therapy.

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Introduction

Glioma therapy is difficult due to the invasive growth that interferes with the total removal of the tumors. Thus, adjuvant therapy, including radiation, various chemotherapeutic agents and biological-response modifiers such as interferon (IFN), is essential for treating malignant gliomas (1,2). Nitrosoureas are alkylating agents that cause cell death by binding to DNA. Among nitrosoureas, 1-(4-amino-2-methyl-5-pyrimidynyl) methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride (ACNU) has been widely used in Japan to treat gliomas due to its ability to permeate the blood-brain barrier and its considerable clinical effects in combination with radiation and IFN-ß until the entry of temozolomide (TMZ) (3). Pt-compounds such as cis-platinum (CDDP) and carboplatin (CBDCA) have also been used for primary and recurrent gliomas in some institutes instead of ACNU because of the severe myelosuppression caused by ACNU and the clinically obvious resistance to it (2).

Although many clinical trials had been performed using adjuvant therapy for malignant gliomas, there has been little evidence based on prospective randomized trials other than an increase in survival by radiation therapy and tumor regression with radiation in combination with nitrosoureas (4). Chemotherapy has not significantly prolonged survival in patients with malignant gliomas. TMZ, an oral alkylating agent, has demonstrated the first level 1 evidence for the survival of glioblastoma patients (5). In Japan, TMZ was approved in July 2006 and TMZ + radiation therapy has become the standard therapy for malignant gliomas.

Drug-resistance genes are some of the most important elements of tumors themselves in determining drug-resistance and O^6-methylguanine-DNA methyltransferase (MGMT) is a drug-resistance gene for nitrosourea (6,7). The cross-linking of

double-stranded DNA by alkylating agents is inhibited by the cellular DNA-repair protein MGMT. MGMT rapidly reverses alkylation at the O^6 position of guanine, thereby averting lethal cross-linking (8). Furthermore, MGMT plays a major role in interfering with the effect of TMZ (9). We performed individual adjuvant therapy (IAT) that was individualized based on the results of reverse transcription-polymerase chain reaction (RT-PCR) for MGMT to treat malignant gliomas in a non-randomized trial (7). Previously, in order to improve IAT, we applied real-time quantitative RT-PCR to the quantitation of MGMT mRNA in IAT for malignant gliomas (10,11).

Patients and methods

Real-time quantitative RT-PCR using SYBR-Green I. MGMT mRNA in 38 neuroepithelial tumor frozen tissues (16 anaplastic astrocytomas, 4 anaplastic oligodendroglial tumors and 18 glioblastoma multiformes) was quantitated immediately after the operation by real-time RT-PCR using SYBR-Green I dye (12). Normal brain and necrotic tissues were excluded from the specimen for analysis. Real-time RT-PCR using SYBR-Green I dye was basically performed, as described previously (10,13). Briefly, total RNA was extracted from frozen tissue specimens weighing ~1 g with Isogen (Nippon Gene, Toyama, Japan). After ethanol precipitation, about 100 μg of total RNA was extracted. Complementary deoxyribonucleic acid (cDNA) (40 μl) solution was synthesized from 2 μg of total RNA with 40 units of a reverse-transcriptase, RAV-2, 54 units of a ribonuclease inhibitor, 16 μl each of a 2.5 mM dNTP mixture, 2.0 μl of 50 pM random primer, 8.0 μl of 5X RAV-2 buffer and diethylpyrocarbonate-treated distilled water (DW) at 42˚C for 90 min. All enzymes and buffers for RT-PCR were purchased from Takara, Otsu, Japan. DNA amplification was carried out using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and detection was performed by measuring the binding of the fluorescent dye SYBR-Green I to double-stranded DNA (12). The PCR reactions were performed with each pair of oligonucleotide primers [MGMT, 5'-CCTGGCTGAATGCCTATTTC and 5'-GATGAGGATGGGGACAGGATT; human ß2-microglobulin (ß2-MG) was used as an internal control, 5'-TTCT GGCCTGGAGGGCATCC and 5'-ATCTTCAAACCTCCA TGATG]. After cycling, the relative quantitation of MGMT mRNA against an internal control, ß2-MG, was possible using the ΔC_T method (12). An amplification plot of the fluorescence signal versus the cycle number was made. The parameter C_T (threshold cycle) was defined as the fractional cycle number at which fluorescence passed the fixed threshold. The difference (ΔC_T) between the mean values in duplicate samples of MGMT and those of ß2-MG was calculated and the relative quantitation value (RQV) was expressed as $2^{-\Delta C_T}$.

Absolute value of MGMT mRNA measured by real-time RT-PCR using Taq Man probe. The absolute value of MGMT mRNA in 17 neuroepithelial tumor frozen tissues (4 anaplastic astrocytomas, an anaplastic oligodendroglial tumor and 12 glioblastoma multiformes) was measured immediately after the operation by real-time RT-PCR using the Taq Man probe. Normal brain and necrotic tissues were excluded from the specimen for analysis. The methods were basically performed as described previously (11,13). Total RNA was extracted from about 10 mg of the specimen stored in RNA*later* RNA Stabilization Reagent (Qiagen, Hilden, Germany) at 4˚C according to the guanidinium thiocyanate-phenol-chloroform extraction method using Isogen (Wako Junyaku, Osaka, Japan) and was collected from the precipitate in ethanol (14). cDNA was synthesized from 1 μg total RNA. The real-time PCR reaction mixture was prepared using a Taq Man Universal Master Mix (Applied Biosystems), 120 nM of each primer described above (11), 200 nM probe (5'-CGA GCA GTG GGA GGA GCA ATG AGA-3') and 2.5 μl of each cDNA sample. The PCR reaction was performed using a real-time PCR system (ABI PRISM 7700 Sequence Detection System: Applied Biosystems). We monitored glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression levels as a quantitative internal control. The standard curves for MGMT and GAPDH mRNA were generated using 10-fold serial-diluted standard plasmid clones inserted with MGMT or GAPDH PCR products as templates, and the expression level of each mRNA was calculated from the standard curve. For precise quantification, the MGMT mRNA expression level of each sample was normalized using the expression of the GAPDH gene.

Individual adjuvant therapy based on RT-PCR results. Since April 2002, 55 IAT, based on the results of real-time RT-PCR for MGMT mRNA, were performed on primary malignant glioma patients as shown in Fig. 1. These cases consisted of 20 patients with anaplastic astrocytomas (grade III), 5 patients with anaplastic oligodendroglial tumors and 30 patients with glioblastoma multiformes. The patient characteristics, surgical reduction rates, main chemotherapeutic agents and combination therapies are summarized in Table I. The performance status was judged by the Eastern Cooperative Oncology Group (ECOG) criteria (15).

Each of the patients underwent IAT after possible resection of the tumor, except for 4 patients who were diagnosed by stereotactic or endoscopic biopsy. Immediately after the operation, real-time quantitative RT-PCR was performed. IAT was approved by the Ethics Committees of Kitasato University and Tokyo Medical University and written informed consent was obtained from each patient before entry into the IAT study.

According to our previously reported retrospective analysis, 42 tumors with an RQV of MGMT <1 in the SYBR-Green I method or an absolute value of MGMT mRNA <6000 copies/μg RNA in the Taq Man probe method were treated with 90-100 mg/m^2 of ACNU (10,11). In 38 therapies, two doses of 1 mg/m^2 of vincristine were used in combination with ACNU. Thirteen tumors with an RQV of MGMT >1 in the SYBR-Green I method or an absolute value of MGMT mRNA >6000 copies/μg RNA in the Taq Man probe method were treated by the administration of cisplatin (20 mg/m^2) for 5 consecutive days in 9 patients or by a single dose of 350-400 mg/m^2 of CBDCA in 4 patients. In 12 of the 13 therapies, the administration of etoposide (60 mg/m^2) for 5 consecutive days was used in combination with platinum compounds (2). IFN-ß (6x10^6 IU) was administered three times a week for 6 weeks in 47 of the

atient characacteristics and the therapeutic modality of individual adjuvant therapy.

Histology	Anaplastic astrocytoma	Anaplastic oligodendroglial tumor	Glioblastoma multiforme	All
No. of patients	20	5	30	55
Age (Mean ± SD)	43.1±14.5	42.2±9.8	45.6±15.3	44.3±14.6
Sex (male %)	60	100	66.7	67.3
Performance status (Mean ± SD)	1.11±1.0	0.60±0.49	1.1±0.93	1.0±0.93
Surgical reduction rate (Mean ± SD)	55.8±27.6	54.0±31.8	77.3±18.0	67.3±25.8
Real-time RT-PCR SYBR-Green I	16	4	18	38
Taq Man probe	4	1	12	17
ACNU-based chemotherapy	13	5	24	42
Vincristine with ACNU	10	4	24	38
Platinum-based chemotherapy	7	0	6	13
Etoposide with platinum compounds	7	0	5	12
Use of interferon-ß	18	4	25	47
Radiation therapy	17	5	29	51
Maintenance therapy	12	4	22	38

SD, standard deviation; ACNU, 1-(4-amino-2-methyl-5-pyrimidinyl) methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride.

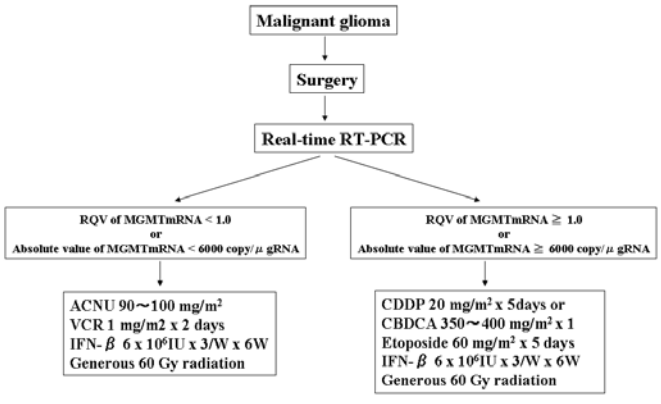

Figure 1. Individual adjuvant therapy for malignant gliomas based on the results of real-time reverse-transcription polymerase chain-reaction (RT-PCR). RQV, relative quantitation value; MGMT, O^6-methylguanine-DNA methyltransferase; ACNU, 1-(4-amino-2-methyl-5-pyrimidynyl) methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride; VCR, vincristine; IFN-ß, interferon-ß, CDDP, cis-platinum and CBDCA, carboplatin.

overall 55 IAT since it has less adverse effects than chemotherapeutic agents and an expected indirect antitumor effect, especially during maintenance therapy (16). Radiation therapy (60 Gy for most patients) was used concurrently in 51 primary chemotherapies after the first operation. Maintenance therapy was performed in 38 patients mainly with weekly or bi-weekly IFN-ß. ACNU or CBDCA was also used once for 2 or 3 months for patients with glioblastoma multiforme in maintenance therapy. Seventeen patients could not receive maintenance therapy as their condition had worsened or due to social reasons.

The extent of the resection was calculated from magnetic resonance imaging (MRI) scans within 3 postoperative days as reported previously (17,18). Enhanced lesions were compared. In non-enhanced tumors, areas of high signal intensity on FLAIR MR images were evaluated. A 95-100% resection was defined as 95, 75-95% was defined as 75% and 50-75% was considered 50%. The effect of IAT was evaluated at least two months after the beginning of therapy according to the Response Evaluation Criteria In Solid Tumors (RECIST criteria) (19). If the tumor had not been enhanced with a contrast medium, the tumor size was evaluated by the high signal intensity area in FLAIR image on MRI. Complete tumor regression was defined as complete response (CR), >30% tumor reduction was defined as partial response (PR), <30% reduction was defined as stable disease (SD) and enlarged tumor mass was considered progressive disease (PD). When there was no residual tumor after total removal, it was described as not evaluable (NE). Although our observation period was too short to evaluate the effect of IAT on survival, analyses were performed regarding the survival period and the progression-free survival (PFS) period. Each reported P-value is two-tailed. All statistical analyses were performed using Stat View 5.0.

Results

Individual adjuvant therapy. IAT, based on the results of real-time RT-PCR of MGMT mRNA, was performed on 55 patients with malignant gliomas. No lethal adverse events that were Grade 4 in the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0 (NCI-CTCv3.0) were noted in any of the 55 patients. The granulocyte-colony stimulating factor was used in 18 patients because of granulocytopenia <1000/mm^2 (Grade 3 of NCI-CTCv3.0). None of the patients had received thrombocyte transfusion. Although an increase in body

Table II. Results of individual adjuvant therapy based on the results of real-time RT-PCR.

Histology	No. of patients	ACNU-based therapy	Effect (CR + PR, %)	Progression free survival			Overall survival		
				Median (M)	6 months (%)	1 year (%)	Median (M)	1 year (%)	2 year (%)
Anaplastic atrocytoma	20	65.0	60.0	10.0	70.0	49.1	25.5	89.5	61.1
Anaplastic oligodendroglial tumor	5	100.0	80.0	10.9	80.0	53.3	23.1	100.0	75.0
Glioblastoma multiforme	30	80.0	40.9	7.6	71.4	50.4	21.7	88.6	70.9

ACNU, 1-(4-amino-2-methyl-5-pyrimidinyl) methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride; CR, complete response and PR, partial response.

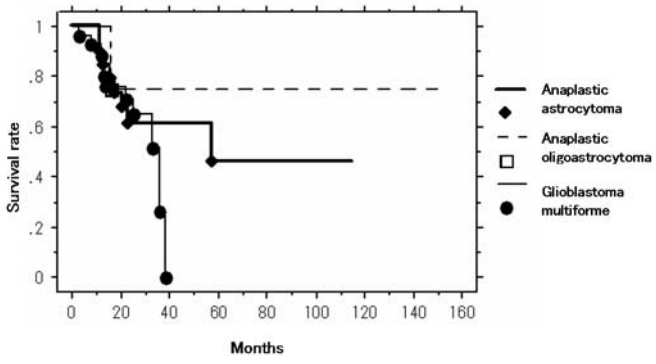

Figure 2. Kaplan-Meier survival curves of patients treated by IAT.

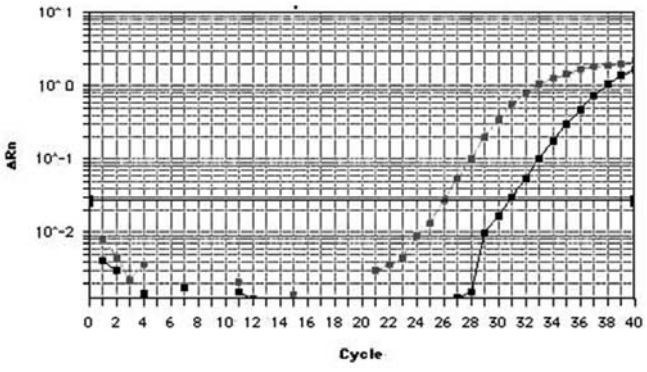

Figure 3. Amplification plot of the fluorescence signal versus the cycle number in real-time RT-PCR using the SYBR-Green I method for MGMT mRNA of an anaplastic astrocytoma specimen from a 54-year old woman. The relative quantitation value of MGMT mRNA in the specimen by real-time RT-PCR was 36. (□, MGMT; ■, ß2-MG).

temperature to >38˚C was observed in almost all of the patients at the first administration of IFN-ß, it was well controlled by antipyretic agents.

IAT, based on the results of real-time RT-PCR, is summarized in Table II. The response rate (CR + PR ratio) was 40.9% for glioblastoma multiformes, 60.0% for anaplastic astrocytomas, 80.0% for anaplastic oligodendroglial tumors and 53.2% for all evaluable therapies. Fig. 2 shows Kaplan-Meier survival curves of the patients treated by IAT. The

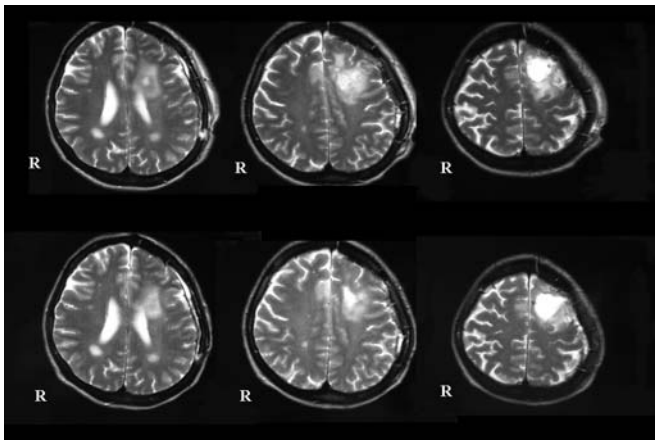

Figure 4. A 54-year-old woman with primary left frontal anaplastic astrocytoma had 50% of the tumor resected under language mapping with chronic subdural electrode placement (upper, immediately after the operation). We started IAT with 5 consecutive days of 30 mg cis-platinum with 100 mg etoposide followed by $6x10^6$ IU of IFN-ß 3 times a week for 5 weeks in combination with generous 60 Gy radiation therapy. She made a partial response, with the tumor reducing 2 months after the beginning of IAT (lower, 2 months after adjuvant therapy).

two-year survival rate of 30 glioblastoma patients was 70.9% and the 1-year progression-free survival rate of glioblastoma patients was 50.4%.

Forty-two patients who had little MGMT mRNA expression mainly received ACNU as IAT and the response rate to therapy was 50.0%. However, 13 patients who had a much greater MGMT mRNA expression received platinum compounds. Platinum-based therapies were considered to be effective in 7 of the 11 evaluable therapies (response rate of 63.6%). The difference between these initial effects of ACNU-based chemotherapy and those of platinum-based chemotherapy was not significant (P=0.5046 by Fisher's exact probability test).

Illustrative cases. A 54-year-old woman with primary left frontal anaplastic astrocytoma had 50% of the tumor resected under language mapping with chronic subdural electrode placement. Immediate postoperative real-time RT-PCR using the SYBR-Green I method showed that the relative quantitation value of MGMT compared to ß2-MG was 36 (Fig. 3). We

IAT with 5 consecutive days of 30 mg CDDP with toposide followed by 6x10^6 IU of IFN-ß 3 times a week for 5 weeks in combination with synchronous generous 60 Gy radiation therapy. She showed a partial response, with the tumor reducing 2 months after the beginning of IAT, as shown in Fig. 4 and has survived >30 months under maintenance therapy with IFN-ß once every 2 weeks.

Discussion

Some authors, including us, have reported that MGMT expression is closely correlated with the clinical or experimental resistance of brain tumors to ACNU (6,7,10). The expression level in DNA measured by PCR may not be always correlated to the enzyme activity. MGMT activity itself seems to be more strongly correlated with clinical resistance to nitrosoureas than mRNA expression. MGMT expression was investigated with regard to its activity about 15 years ago (20). However, the quantitation of MGMT activity is complicated and requires too much time for clinical use. Real-time RT-PCR is a simple, rapid and clinically applicable method for determining nitrosourea resistance (10). According to our previous results, a tumor with a compensated MGMT mRNA value >6000 copies/μg RNA in real-time quantitative RT-PCR should not be treated by ACNU (11).

Although PCR has generally not been considered to be suitable for quantification (13), quantification by PCR has been attempted because it is easy, rapid and sensitive. At present, real-time PCR seems to be the most sensitive and rapid method for the quantitation of DNA and RNA (21). We used SYBR-Green I dye, which binds to double-stranded DNA and provides a fluorescent signal (10,12). This approach is simpler and more sensitive, since many fluorescent labels are incorporated into amplified fragments, instead of just one molecule. The disadvantage of fluorescence dye is that specific and non-specific products generate a signal. In the present study, an original Taq Man probe was used for absolute quantitation by real-time PCR. Fluorescently labeled probes for detecting amplified products have been used in most studies on real-time PCR for diagnostic purposes (22). Moreover, normalization of MGMT mRNA by the amount of the internal control, GAPDH mRNA, gives further useful information for the clinical use of nitrosoureas (11).

Our therapeutic concept regarding IAT is to avoid using nitrosoureas in MGMT active tumors. There are other approaches that overcome the resistance of MGMT to nitrosoureas. MGMT is different from other enzymes because it is consumed. Some authors have tried to repair alkylated DNA by MGMT using this specificity. Excessive alkylating agents, such as high-dose ACNU or 5-(3-3,-dimethyl-1-triazeno) imidazole-4-carboxamate, which can waste MGMT, have been used for this purpose (23,24). However, the results of these therapies were disappointing due to severe side effects and a low response rate. Antisense MGMT mRNA and some other drugs may reduce the ability of MGMT to repair the effects of alkylation (25). Although O^6-benzylguanine and streptozocin have been expected to modulate the resistance to nitrosoureas, favorable clinical results with large numbers of patients have not yet been reported (26,27). Our previous study suggested that platinum compounds might play a role in the down-regulation of MGMT mRNA expression and the up-regulation of the sensitivity to ACNU (28). The down-regulation of MGMT activity by TMZ has also been reported (29).

Previously, the inactivation of MGMT by promoter hypermethylation has been reported to be a common event in the primary human neoplasm (30). Esteller *et al* reported that hypermethylation of the MGMT promoter in gliomas was a useful predictor of the responsiveness of the tumors to alkylating agents (31,32). Our results of quantitative RT-PCR for MGMT showed a close relationship between MGMT mRNA expression and the prognosis of patients treated with ACNU. This does not contradict the promoter methylation theory of MGMT inactivation because promoter hypermethylation resulted in a decrease or disappearance of MGMT expression in real-time quantitative RT-PCR. Methylation-specific PCR is not quantitative and it has been suggested that this method gives vague results. Methylated and unmethylated bands are often seen in the same tumor specimen.

Evidence-based medicine (EBM) now seems to be widely accepted (33). In adjuvant glioma therapy, there has been no confirmed evidence that any treatment other than radiation therapy prolonged patient survival (4). A large randomized trial by the European Organization for Research and Treatment of Cancer of newly diagnosed patients with glioblastoma compared the results with radiation alone to those with radiation + TMZ. This report by Stupp *et al* constituted the first evidence of level 1 in chemotherapy of glioblastoma and in particular that radioactive rays + TMZ may be a normal treatment for glioma in the future (5). In contrast, one of the major reasons why glioma therapy has been difficult is the heterogeneity of the tumor. Histological features, biological behavior and the response to therapy all vary in such cases. Histological grading does not always coincide with the sensitivity of the tumor. A new grouping for glioma that indicates the appropriate therapeutic modality is needed. The so-called 'made to order' therapy such as our IAT appears to still be effective for glioma.

TMZ was released in Japan in September 2006. TMZ has already been used in many cases in Europe and America and good treatment results have been achieved. In Japan, treatment with TMZ is also expected to give improved results. Since MGMT is an enzyme that is thought to confer resistance to TMZ as well as nitrosoureas (34), our technique of ascertaining MGMT activity by real-time RT-PCR may be applicable for the use of TMZ. As previously mentioned, the down-regulation of MGMT activity by TMZ due to hypermethylation has also been reported (29). The daily administration of low-dose TMZ concomitant with radiation therapy followed by adjuvant therapy with nitrosoureas may be effective (29,35).

TMZ is an oral medication with much fewer side effects than conventional anticancer agents and is thought to be extremely useful during maintenance therapy for glioma and recurrence (36,37). Although TMZ is supported by Level 1 evidence, should we really be satisfied with an extension of the duration of survival of 2.5 months in glioblastoma? It seems unlikely that all glioma should be treated by TMZ +

radiation. However, while we should be mindful of the small number of patients, our results with IAT were better than those with TMZ + radiation for glioblastoma.

Our present results with IAT were superior to those with TMZ + radiation for glioblastoma. In our results, the survival benefit of IAT for anaplastic astrocytoma was identical to that for glioblastoma because of relative short follow-up periods. IAT, based on the results of real-time RT-PCR, may lead to a beneficial glioma therapy compared to TMZ-radiation therapy. A prospective randomized multi-center study should be performed to confirm the efficacy of this new method of glioma therapy.

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