Long-term therapy with temozolomide is a feasible option for newly diagnosed glioblastoma: a single-institution experience with as many as 101 temozolomide cycles

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Object. The objective of this study was to report the authors' experience with the long-term administration of temozolomide (TMZ; > 6 cycles, up to 101) in patients with newly diagnosed glioblastoma and to analyze its feasibility and safety as well as its impact on survival. The authors also compared data obtained from the group of patients undergoing long-term TMZ treatment with data from patients treated with a standard TMZ protocol.

Methods. A retrospective analysis was conducted of 37 patients who underwent operations for glioblastoma between 2004 and 2012. Volumetric analysis of postoperative Gd-enhanced MR images, obtained within 48 hours, confirmed tumor gross-total resection (GTR) in all but 2 patients. All patients received the first cycle of TMZ at a dosage of 150 mg/m² starting on the second or third postsurgical day. Afterward, patients received concomitant radiochemotherapy according to the Stupp protocol. With regard to adjuvant TMZ therapy, the 19 patients in Group A, aged 30–72 years (mean 56.1 years), received 150 mg/m² for 5 days every 28 days for more than 6 cycles (range 7–101 cycles). The 18 patients in Group B, aged 46–82 years (mean 64.8 years), received the same dose, but for no more than 6 cycles. O⁶-methylguanine-DNA methyltransferase (MGMT) promoter methylation status was analyzed for both groups and correlated with overall survival (OS) and progression-free survival (PFS). The impact of age, sex, Karnofsky Performance Scale score, and Ki 67 staining were also considered.

Results. All patients but 1 in Group A survived at least 18 months (range 18–101 months), and patients in Group B survived no more than 17 months (range 2–17 months). The long-term survivors (Group A), defined as patients who survived at least 12 months after diagnosis, were 51.3% of the total (19/37). Kaplan-Meier curve analysis showed that patients treated with more than 6 TMZ cycles had OS and PFS that was significantly longer than patients receiving standard treatment (median OS 28 months vs 8 months, respectively; p = 0.0001; median PFS 20 months vs 4 months, respectively; p = 0.0002). By univariate and multivariate Cox proportional hazard regression analysis, MGMT methylation status and number of TMZ cycles appeared to be survival prognostic factors in patients with glioblastoma. After controlling for MGMT status, highly significant differences related to OS and PFS between patients with standard and long-term TMZ treatment were still detected. Furthermore, in Group A and B, the statistical correlation of MGMT status to the number of TMZ cycles showed a significant difference only in Group A patients, suggesting that MGMT promoter methylation was predictive of response for long-term TMZ treatment. Prolonged therapy did not confer hematological toxicity or opportunistic infections in either patient group.

Conclusions. This study describes the longest experience so far reported with TMZ in patients with newly diagnosed glioblastomas, with as many as 101 cycles, who were treated using GTR. Statistically significant data confirm that median survival correlates with MGMT promoter methylation status as well as with the number of TMZ cycles administered. Long-term TMZ therapy appears feasible and safe.
(http://thejns.org/doi/abs/10.3171/2014.9.FOCUS14502)

Key Words • complications • high-grade glioma • glioblastoma • long-term administration • temozolomide • toxicity

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Glioblastoma is the most common and malignant brain tumor, with an estimated annual incidence of 3 million new cases worldwide.[11] Its prognosis remains poor, with an overall patient survival ranging between 12 and 18 months,[7] a 2-year survival rate of 26.5%, and a 70% recurrence rate within 1 year from initial treatment,[22] despite the advancements in treatment modalities

achieved over the last decade and the extensive research on related pathological, immunohistochemical, and genetic features.[1,12,18,20] The impact of both gross-total resection (GTR)[14,15,21] and chemotherapy with temozolomide (TMZ)[22] on patient survival has been demonstrated. In particular, following the randomized Phase III trial by Stupp et al., the current standard, postsurgical, first-line treatment for newly diagnosed glioblastoma is based on concurrent radiation therapy and TMZ chemotherapy followed by 6 cycles of adjuvant TMZ therapy, i.e., the Stupp protocol.[22] Temozolomide treatment has been associated with significantly better tolerability and fewer side effects than other drugs previously used for chemotherapy in patients with glioblastoma.

The role of O^6-methylguanine-DNA methyltransferase (MGMT) promoter methylation has been studied and associated with a better outcome in patients with glioblastoma undergoing TMZ chemotherapy,[5,7] and analysis of MGMT promoter methylation status has become standard in patients with newly diagnosed glioblastoma.[17]

Despite anecdotal experiences with TMZ treatment longer than 6 cycles, very limited data are currently available in the literature,[6,8,10,16] and only 2 papers—even if with important bias—described the results of long-term TMZ treatment in cohorts of patients with glioblastoma.[6,16] In this paper we report our experience on the long-term administration of TMZ in a series of primary glioblastomas, analyzing its impact on patients' progression-free survival (PFS) and overall survival (OS), and compare these data with PFS and OS of a matched cohort of patients with primary glioblastoma treated according to the standard Stupp protocol.

Methods

Study Population

Of all patients who underwent surgery for newly diagnosed glioblastoma from 2004 to 2012, 37 patients were included in this study, consisting of 19 males and 18 females and ranging in age from 30 to 82 years (mean 60.7 years). In particular, we analyzed those patients who received more than 6 cycles of TMZ chemotherapy (Group A) and compared them with a matched cohort of patients receiving no more than 6 TMZ cycles (Group B). Nineteen patients (10 males), with a mean age of 56.1 years (range 30–72 years) were included in Group A, and 18 patients (9 males), with a mean age of 64.8 years (range 46–82 years), in Group B. All patients underwent surgery for primary glioblastoma with the aid of neuronavigation, and all but 2 patients received tumor GTR, as documented by Gd-enhanced MRI performed within 48 hours after surgery. Moreover, in all cases GTR was verified using volumetric MRI evaluation according to the criteria proposed by Sanai et al.:[15]

(preoperative tumor volume − postoperative tumor volume)/ postoperative tumor volume × 100

Gross-total resection was defined as the absence of solid enhancing tissue on postoperative MRI.

One radiologist (Dr. Palmucci) blinded to clinical outcome reviewed all patients' preoperative and early postoperative MR images. These images were processed on a dedicated workstation (Advantage Workstation VolumeShare 5, GE HealthCare). Volumetric measurements were performed on Gd-enhanced, T1-weighted, 3D fast spoiled gradient echo sequences, applying a volume-rendering segmentation technique. The radiologist traced manual contour of the tumor lesion on axial images; in addition, 2D maximal length and short axis were automatically generated for each lesion.

To increase tumor extent of resection (EOR), starting in 2010 5-aminolevulinic acid (5-ALA) was routinely used at our institution (Department of Neurosurgery, Policlinico "G. Rodolico" University Hospital) during surgery for suspected high-grade glioma; it was used in 15 (40.5%) of 37 patients enrolled in this study, including 5 patients in Group A (26.3%) and 10 patients in Group B (55.6%). According to early postoperative volumetric MRI all but 2 patients received tumor GTR, including 1 each in Group A and Group B who underwent subtotal resection (STR), and regardless of whether 5-ALA had been used, no significant differences were observed between the two groups. In 1 of the 2 patients with STR, 5-ALA had been used during the first surgery, and despite the surgeon's intraoperative perception of GTR, postoperative volumetric MRI demonstrated a residual tumor nodule.

TMZ Protocol

Postoperatively, after informing patients and their close relatives about known pros and cons of TMZ chemotherapy, if the intraoperative histological diagnosis was of likely high-grade glioma, and such information was also consistent with the surgeon's anatomosurgical perception, TMZ administration was started on an early basis, usually on the second or third postoperative day, at a dose of 150 mg/m² for 5 days. Following this first TMZ cycle, all patients received radiation therapy and concurrent TMZ administration dosed according to the Stupp protocol. For adjuvant TMZ therapy, patients in Group A received 150 mg/m² for 5 days every 28 days for more than 6 cycles (up to 101), whereas those in Group B were treated with the same adjuvant TMZ dose regimen for no more than 6 cycles.

Before starting to use the long-term TMZ treatment protocol we did not identify specific factors or patient-related features supporting our decision to continue TMZ therapy for more than 6 cycles; early postoperative MRI-proven tumor GTR, good clinical condition (according to Karnofsky Performance Scale [KPS] score) both after surgery and during follow-up, absence of both TMZ-related toxicity and of disease progression (i.e., complete response according to the Macdonald criteria[9]), as well as the patient's willingness to continue TMZ therapy were the important elements supporting our decision to continue to administer TMZ. As a consequence, we did not identify factors other than TMZ-related side effects or disease progression with clinical deterioration as potential contraindications to TMZ long-term therapy. Tumor location, extent of edema, specific pathological features, and MGMT methylation status were not initially considered factors supporting or not supporting the choice to perform such long-term chemotherapy with TMZ. If a

disease-complete response was confirmed according to Macdonald criteria,[9] the patient's KPS score did not decrease, and monthly blood tests ruled out TMZ-related hematological toxicity, then TMZ therapy was continued.

The above strategy, which applied to patients included in Group A, was agreed upon and carried out by the neurosurgical team even during patients' follow-up evaluations. Conversely, Group B patients were managed by the oncology team according to the Stupp protocol. In these patients adjuvant TMZ therapy was stopped either after 6 cycles or because of proven disease progression with clinical deterioration.

Patients were assessed 4 weeks after surgery and then every 3 months. However, before starting each TMZ cycle, blood tests were performed to screen for hematological toxicity. Every 3 months follow-up MR images were obtained to assess the radiological response to TMZ therapy. Over the years, we commonly evaluated Gd-enhanced, T1-weighted MRI sequences looking for recurrent tumor. Recently, all scans were reviewed retrospectively according to the updated Response Assessment in Neuro-Oncology (RANO) criteria,[25] with the aim to also analyze T2-weighted and fluid-attenuated inversion recovery (FLAIR) images and investigate signs of progression of the nonenhancing tumor component. Immunohistochemical analysis for Ki 67 expression as well as MGMT methylation status was performed retrospectively on all operated cases.

Immunohistochemical Analysis

Immunohistochemical analysis was performed on 5-μm–thick, formalin-fixed, paraffin-embedded tissue sections using MIB-1, a monoclonal antibody directed against the Ki 67 antigen (1:75, Dako Corporation). Immunohistochemical studies were performed with the labeled streptavidin-biotin peroxidase detection system using the Ventana automated immunostainer (Ventana Medical Systems).

Briefly, the sections were deparaffinized and dehydrated in a series of "dewax" solutions and alcohol. Heat-induced antigen retrieval was performed with a high pH antigen retrieval buffer (ER2). After washing with phosphate-buffered saline 3 times for 5 minutes each, the slides were incubated with 0.5% H_2O_2 for 20 minutes to quench endogenous peroxidase. Normal horse serum at a dilution of 1:50 was added to each slide to block nonspecific reactions and incubated for 20 minutes. Incubation with primary antibody was followed by incubation with a biotinylated rabbit secondary antibody at a dilution of 1:200 for 45 minutes and 3 washes with phosphate-buffered saline and substrate. The positive results were visualized with 3,3'-diaminobenzidine. Finally, the sections were counterstained and coverslipped. Any nuclear staining was considered as indicative of Ki 67 expression. The control slide (without the primary antibody) was used to ensure the protocols were followed correctly.

Staining Interpretation

The Ki 67 labeling index was evaluated in the highest immunoreactivity fields. It was expressed as a percentage and was determined by dividing the number of positive staining nuclei by 1000 tumor cells. The quantitative analysis of this staining was performed with the software-aided quantification of Ki 67 immunoreactivity. Five fields, randomly selected from each section, were analyzed and the percentage of area stained with the Ki 67 antibody was calculated using image analysis software (AxioVision release 4.8.2, SP2 Software, Carl Zeiss Microscopy GmbH). Digital photomicrographs were taken using the Zeiss Axioplan (Carl Zeiss) light microscope, using an objective lens with magnification ×20 (i.e., final magnification ×200), fitted with a digital camera (AxioCam MRc5, Carl Zeiss). Evaluations were made by two blinded investigators (R.C. and F.M.), whose evaluations were assumed to be correct if values were not significantly different.

DNA Isolation

DNA was isolated by macrodissection from 3-μm sections of formalin-fixed, paraffin-embedded tumor tissue samples and processed with the QIAamp DNA FFPE Kit (Qiagen) following the manufacturer's instructions. DNA quality control and yield were assessed by spectrophotometry using a Nanodrop machine (Thermo Scientific).

Bisulfite Treatment and MGMT Methylation Analysis

Three hundred nanograms of genomic DNA samples and universal polymethylated DNA (EpiTect Positive Control DNA Set, Qiagen) was subjected to bisulfite conversion with the EpiTect bisulfite kit (Qiagen) according to the manufacturer's instructions. Bisulfite-treated DNA, in addition to unmethylated DNA from controls, was analyzed to determine the methylation status of the CpG island of MGMT promoter methylation status by pyrosequencing analysis. In particular, 4 CpG sites of the MGMT promoter (exon 1, Ensembl ID: OTTHUMT00000051009) were analyzed using a PyroMark 24 system (Qiagen), according to the manufacturer's protocol. Templates for pyrosequencing were amplified with primers that were biotinylated for template strands (MGMT PyroMark CpG Assay kit, Qiagen). The biotinylated polymerase chain reaction (PCR) products were then immobilized on streptavidin-coated Sepharose beads (GE Healthcare), and the single-stranded DNA templates were analyzed by PyroMark Q24 (Qiagen). Subsequent quantification of the methylation density for the 4 investigated CpG sites was performed using the PyroMark Q24 software (Fig. 1).

To define cases with methylated versus unmethylated MGMT promoter, the average percentage value of the methylation percentage obtained at each of the investigated CpG dinucleotides was calculated. Cases with an average methylation < 9% (mean ± 2 SDs for DNA in controls) were regarded as MGMT promoter "unmethylated," patients with an average methylation of 9%–25% were regarded as MGMT promoter "intermediate methylated," and patients with an average methylation more than 25% were "highly methylated," as previously described.[4]

In addition to pyrosequencing analysis, we determined MGMT methylation by methylation-specific PCR
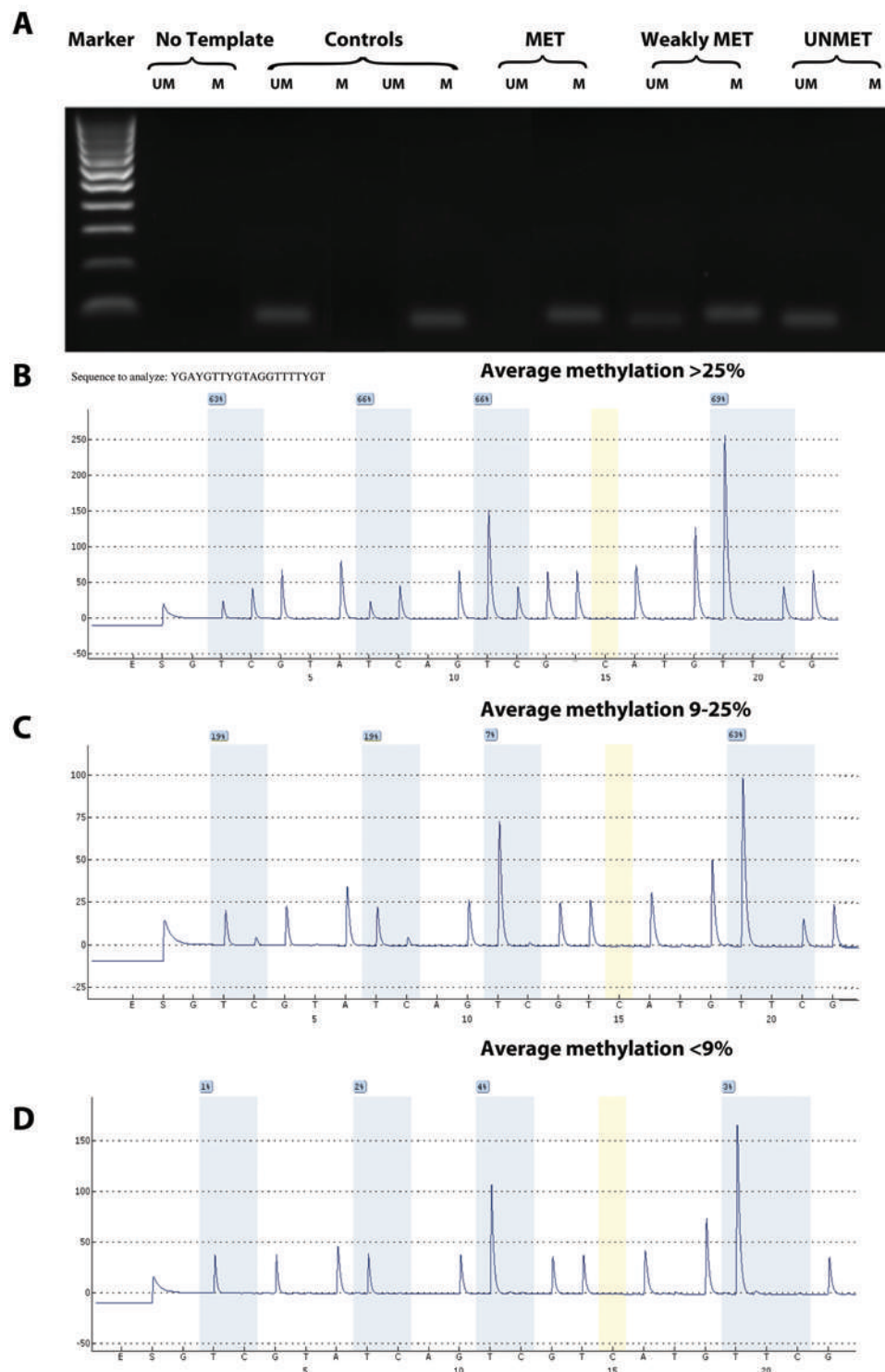
Fig. 1. Schematic representation of the MGMT promoter methylation methodology. **A:** Example results obtained by MSP for unmethylated (UM) and methylated (M) promoter sequences in no template control, unmethylated DNA control, universal polymethylated DNA control, and tumor samples. **B–D:** Allelic MGMT methylation analysis by pyrosequencing of sodium bisulfite–modified DNA extracted from gliomas methylated (> 25%, B), intermediate methylated (9%–25%, C) and unmethylated (< 9%, D). The methylation percentage at the individual CpG sites is noted in the *blue boxes* on top of the pyrogram. The *yellow bar* represents the internal control for the completion of bisulfite conversion. Values on the x-axes represent the number of analyzed bases.

analysis (MSP). MSP assay was performed using a 2-step nested PCR approach as previously described.[5,7] The MSP reactions were performed in 25 μl by 2720 Thermal Cycler Applied Biosystem PCR. Universal unmethylated and polymethylated DNA were included as controls in each set of reactions, in addition to a negative control sample without DNA. The PCR products were resolved on 4% low-melting-point agarose gels (Fig. 1).

The analysis of MSP data was performed by investigators who were blinded to clinical data (Drs. Paratore and Caltabiano). Individual tumors showing only very weak PCR products for the methylated MGMT sequence promoter but strong PCR products for the unmethylated MGMT sequence promoter were judged as "weakly methylated."

Corticosteroid Use

All patients received the same dose of corticosteroids before surgery (4 mg of dexamethasone, 4 times a day every 6 hours); similarly, after surgery the same dosing protocol was initially administered to patients in both groups until gradually tapered and stopped over 1 week. However, when patients started radiation therapy, they received 4 mg of dexamethasone twice a day for 3 weeks; corticosteroids were then reduced to 4 mg daily and eventually stopped over 10–14 days. During follow-up when patients presented because of clinical deterioration secondary to disease progression, corticosteroids were started again according to standard protocol in patients with glioblastoma.

Statistical Analysis

Statistical analysis was performed using SPSS 16.0 software (SPSS, Inc.). The t-test and McNemar's chi-square test were used for comparison of 2 group variables and 2 methylation detection methods. The Kaplan-Meier method was used to estimate OS and PFS, and the log-rank test was used to assess the differences. Overall survival was measured from the time of resection to patient death or the last date when the patient was known to be alive. Progression-free survival was defined as the time from resection to the time of demonstrated tumor growth on follow-up imaging, or evidence of neurological decline. Univariate and multivariate analyses based on a Cox proportional hazard regression model were performed to identify potential prognostic factors for OS and PFS in patients with long-term TMZ therapy.

Results

Epidemiological Data

In total, 37 patients (19 men and 18 women) with only primary glioblastoma were included in this retrospective analysis. A comparison of patient data and clinical features in each group is summarized in Table 1, including sex, age at diagnosis, KPS score, MGMT methylation status, Ki 67 staining, and number of TMZ cycles.

Radiological Evaluation of EOR and of Response to Therapy

The EOR was evaluated comparing preoperative MR images with postoperative examinations obtained within 48 hours after surgery, as well as volumetric MRI evaluation. The preoperative mean tumor volume was 32.20 cm³ (range 0.79–69.4 cm³), with a standard deviation of 19.32; mean 2D maximal length value was 47.4 mm (range 15–69 mm), with a standard deviation of 14.33, whereas mean short axis value was 31.76 mm (range 8–50 mm), with a standard deviation of 9.99. Residual disease (0.50 cm³ and 0. 29 cm³, respectively) was reported in only 2 early postoperative MRI examinations; in these 2 patients (1 each in Group A and Group B), the images revealed hyperintense nodules after Gd administration. These cases were considered STRs. For cases with residual disease, GTR was 98.7% and 98.8%.

Moreover, follow-up MR images were reviewed and retrospectively scrutinized for recurrent disease according to the RANO criteria[25] to identify patients showing signs of nonenhancing tumor progression on T2-weighted or FLAIR images. The RANO criteria were published in 2010, 6 years after the first surgery performed in this study. The latter evaluation confirmed data on tumor progression previously obtained on follow-up MR images. When clinically required, PET scans were also obtained to confirm the diagnosis of recurrent tumor. In particular, 2 patients in Group A and 1 in Group B underwent PET scans, which revealed recurrence in 1 of the 2 Group A patients and radionecrosis in the Group B patient. In all but 6 Group A patients (who are still alive), and all but 1 Group B patient (who survived only 6 months and received 6 TMZ cycles), the last follow-up MR image documented tumor progression, according to Macdonald's criteria.[9]

KPS Score

Patients in Group A had a preoperative KPS score ranging from 40 to 100 (mean score 71.5); postoperative KPS score at the 3-month evaluation ranged between 50 and 100 (mean score 76.3). In Group B, the preoperative KPS score ranged from 40 to 80 (mean 62.2). Three months after surgery, the KPS score in this group was between 30 and 90, with a mean of 65.5 (the patient with the lowest score had a postsurgical intracerebral hematoma).

Ki 67 Immunohistochemical Analysis

Ki 67 expression was low (< 20%) in 26 tumors (70%; Fig. 2 upper) and high (≥ 20%) in 11 tumors (30%; Fig. 2 lower). No significant difference in OS and PFS was observed between patients with low or high Ki 67 expression (OS, p = 0.605, log-rank test; PFS, p = 0.928, log-rank test). No statistically significant differences related to OS and PFS between patients with long-term and standard TMZ treatment were detected when controlling for Ki 67 expression levels. Therefore, high Ki 67 levels were not predictive of response in long-term TMZ therapy (Group A, p = 0.612, and Group B, p = 0.743; Student t-test).

MGMT Methylation Analysis

Quantitative data on the percentage of MGMT promoter methylated alleles were obtained by pyrosequencing analysis. Using the average methylation of less than

TABLE 1: Demographics and baseline characteristics of patients with glioblastoma who underwent long-term (Group A) or standard TMZ treatment (Group B)

Variable	Group A	Group B	p Value*
no. of patients	19	18	
sex			
males	10	9	
females	9	9	
mean age ± SD (yrs)	56 ± 11	65 ± 11	0.022
mean KPS score ± SD	72 ± 15	62 ± 14	0.058
MGMT status			
methylated and partly methylated	14	5	
unmethylated	5	13	0.005
mean Ki 67 ± SD (%)	15 ± 12	19 ± 13	0.317
mean no. of TMZ cycles ± SD	27 ± 26	4 ± 2	0.001

* The t-test was used for all comparisons except MGMT status unmethylated (chi-square test).

9% as the unique cutoff to cluster patients based on MGMT methylation status, 18 (49%) of 37 glioblastomas were scored as unmethylated and 19 (51%) as methylated. When the average methylation range between 9% and 25% was also considered, the methylated cases were divided into 2 other subsets: 9 glioblastomas (26.7%) displaying 9%–25% methylated alleles were defined as intermediate methylated, and 10 glioblastomas (24.3%) with more than 25% methylated alleles were defined as

Fig. 2. Low Ki 67 expression (5%; **upper**) and high Ki 67 expression (60%; **lower**) in 2 patients with glioblastoma. Original magnification ×200.

methylated. The results of methylation MGMT promoter status by pyrosequencing were confirmed using MSP assay. All gliomas samples lacking MGMT methylation (n = 18) by pyrosequencing were also found to be unmethylated using MSP. Likewise, 10 glioblastomas were defined as intermediate methylated, and 5 other tumor samples were considered methylated using both techniques. The remaining 4 tumor samples were scored as methylated on pyrosequencing and weakly methylated on MSP. It should be noted that when comparing the results of the 2 different bisulfite method techniques, they showed a high degree of concordance (p < 0.001, McNemar's chi-square test).

Factors Influencing Outcome in Long-Term Survivors

All patients but 1 in Group A survived at least 18 months (range 18–101 months), and all patients in Group B survived no more than 17 months (range 2–17 months). Overall survival and PFS were greater in Group A than in Group B (p < 0.001 and p = 0.002, respectively), and a significant difference was found between the 2 groups even after controlling for age, i.e., patients ≤ 60 years old compared with those > 60 years old in Group A versus Group B (Table 2).

Considering the whole tumor set, independently of clinical features and MGMT methylation status, the median OS and PFS were 17 months (95% CI 11–22 months) and 10 months (95% CI 6–15 months), respectively. The long-term survivors (Group A), defined as patients who survived at least 12 months after diagnosis, were 19 (51.3%) of the 37 total patients. In Group A, 1 of 19 patients lived 12 months, 5 (26.3%) lived for a period of time ranging from 18 to 24 months, and 13 (68.4%) lived for longer than 24 months (including 1 patient with STR). In Group B, 1 patient lived only 2 months after surgery, 14 (77.8%) lived between 6 and 12 months (including 1 patient with STR), and 3 patients (16.7%) survived 14, 16, and 17 months, respectively.

When PFS was considered in Group A, 2 patients had a PFS of no more than 6 months, 5 patients (26.3%) a PFS between 6 and 12 months, 5 (26.3%) between 13
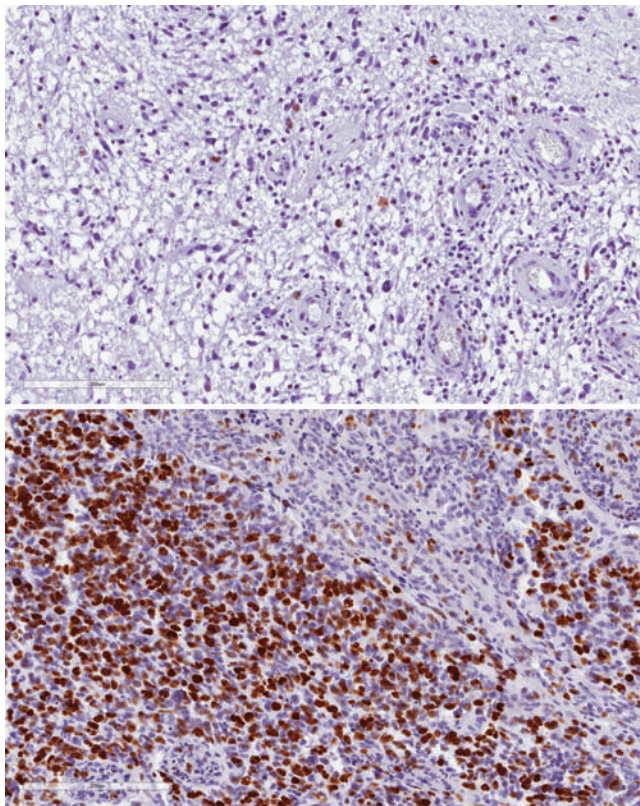
and 24 months, and in 7 cases PFS was longer than 25 months (36.8%, including 1 patient with STR). Conversely, in Group B, 13 patients had a PFS of no more than 6 months (72.2%, including 1 patient with STR), 3 patients (16.7%) a PFS between 6 and 12 months, and only 2 patients (11.1%) more than 12 months.

With regard to the influence of clinical variables such as age, sex, KPS score, Ki 67 expression, and MGMT methylation status on OS and PFS in patients with glioblastoma, univariate Cox proportional hazard analysis showed that age, KPS score, and MGMT status were potentially prognostic factors ($p < 0.05$; Table 3). By focusing on MGMT methylation status, Kaplan-Meier curve analyses indicated that patients with methylated MGMT promoter (> 9% cutoff) had significant improvement of median OS and PFS (27 months [95% CI 12–40 months] and 12 months [95% CI 7–25 months], respectively) as compared with patients with unmethylated MGMT promoter (11 months [95% CI 8–17 months] and 5 months [95% CI 4–12 months], respectively; Fig. 3A). In addition, statistical analysis comparing OS and PFS to methylation data of 3 different cutoff values (average methylation < 9%, 9%–25%, and > 25%) also showed that patients with methylated and partly methylated MGMT promoter had a longer OS than unmethylated cases, and those with the highest methylation showed the longest OS and PFS (median 34 and 20 months, respectively), suggesting that the extent of methylation affected survival (Fig. 3B and C). After controlling for MGMT status, highly significant differences related to OS and PFS between patients with long-term TMZ treatment were still detected: the p values for OS and PFS in Group A patients with MGMT unmethylated versus methylated were $p = 0.012$ and 0.0181, respectively, and in Group B patients with MGMT unmethylated versus methylated were $p = 0.391$ and 0.14, respectively; Fig. 4A and B).

When the potentially prognostic factors for survival by univariate analysis were included in a multivariate Cox regression hazard model, MGMT status was the only statistically significant variable (Table 3). The MGMT status was a significant predictor for OS ($p = 0.04$) and

was nearly a significant predictor for PFS ($p = 0.06$). When the number of TMZ cycles was also included as a predictive variable in univariate and multivariate analyses, the number of TMZ treatment cycles was an independent predictor for both OS and PFS ($p = 0.002$ and $p = 0.010$, respectively; Table 3). As shown by Kaplan-Meier curve analysis, patients who received long-term TMZ treatment had longer OS and PFS than patients receiving standard treatment (Fig. 5). Furthermore, the statistical comparison of the number of TMZ cycles with MGMT status in Groups A and B showed a significant difference only in Group A patients, suggesting that methylation was predictive of response for long-term TMZ treatment ($p = 0.0001$ for TMZ cycles in Group A patients with MGMT unmethylated versus methylated, versus $p = 0.187$ in Group B; Fig. 4C).

Corticosteroids Use

With regard to the use of corticosteroids pre- and postoperatively, as well as during radiation therapy, there were no preset protocol differences (dose and length of therapy) between patients treated with long-term versus short-term TMZ therapy.

Management of Recurrences

Seven (18.9%) of 37 patients presented with recurrent glioblastoma, including 5 (26.3%) of 19 patients in Group A and 2 (11.1%) of 18 in Group B. Recurrences in Group A were treated by second-line chemotherapy, including TMZ (150 mg/m² for 5 days every 28 days) and fotemustine (75 mg/m² every 21 days) for 3 months in 1 patient, and by surgery followed by further TMZ therapy (also at 150 mg/m²) in the remaining 4 individuals. In Group B, 1 patient underwent second-line chemotherapy using the same protocol detailed above and the other underwent surgery but no further TMZ therapy.

Among these 7 patients with recurrences, 5-ALA had been used only in 2 cases during the first surgery and in both of these patients early postoperative MRI had confirmed GTR. These 2 patients are also the ones who underwent second-line chemotherapy without a second

TABLE 2: Overall survival, PFS, and cycles of TMZ therapy in Group A and Group B patients*

Group	No. of Patients	Age (yrs)	OS (mos)	PFS (mos)	TMZ Cycles
overall					
Group A	19	56 ± 11	36 ± 24	29 ± 26	27 ± 26
Group B	18	65 ± 11	9 ± 4	6 ± 4	4 ± 2
p value		0.022	<0.001	0.002	0.001
age ≤60 yrs					
Group A	10	48 ± 8	41 ± 30	33 ± 33	33 ± 33
Group B	6	45 ± 6	13 ± 3	11 ± 5	8 ± 1
p value		0.323	0.010	0.035	0.022
age >60 yrs					
Group A	9	65 ± 4	32 ± 13	24 ± 14	20 ± 14
Group B	12	72 ± 7	9 ± 4	5 ± 4	4 ± 2
p value		0.012	0.001	0.007	0.011

* All values other than p value are given as mean ± SD. The t-test was used for all statistical comparisons.

TABLE 3: Univariate and multivariate Cox proportional hazards model results for OS and PFS

Variable	OS Univariate	OS Multivariate	PFS Univariate	PFS Multivariate
sex	0.681	—	0.303	—
age	0.032*	0.727	0.015*	0.911
KPS score	0.038*	0.119	0.126	0.702
Ki-67 expression	0.196	—	0.616	—
MGMT status	0.018*	0.039*	0.019*	0.06
no. of TMZ cycles	0.001*	0.002*	0.001*	0.010*

* Statistically significant p values.

surgery following the diagnosis of recurrent disease. With regard to the onset of recurrences in these 7 patients, we observed that the mean time until the diagnosis of recurrent disease was 19.28 months (range 3–42 months); specifically, 24.4 months (range 11–42 months) in the 5 Group A patients and 6.5 months (range 3–10 months) in those included in Group B. Interestingly, in patients treated with long-term TMZ therapy (Group A), recurrences presented at a significantly later stage (24.4 months after first surgery) compared with patients treated using the Stupp protocol (6.5 months, Group B).

Complications

No surgery-related complications were identified other than 1 case of postoperative intracerebral hematoma, which occurred early after surgery in 1 Group B patient. No cumulative toxicity was observed in either group studied. In Group A patients, who received more than 6 cycles of TMZ, only minor side effects were observed and these were not clinically significant, such as platelet reduction in 2 patients and white cell reduction in other 2 patients. These side effects did not require treatment, and TMZ administration was not interrupted. In all patients, monthly blood tests ruled out other clinically relevant blood toxicity. Nausea and vomiting were rarely reported, regardless of the group, and were mild and self-limiting. TMZ treatment was never stopped because of such side effects.

Discussion

The current standard TMZ treatment for newly diagnosed glioblastoma is based on administration of 1 concurrent and up to 6 adjuvant cycles of TMZ, at the dose of 75 mg/m^2 and 150–200 mg/m^2, respectively (i.e., the Stupp protocol).[22] Safety and tolerability profiles of TMZ have been widely demonstrated,[23] and the Stupp protocol is currently considered the standard of care for first-line chemotherapy treatment in newly diagnosed cerebral glioblastomas. However, despite such treatment the prognosis for glioblastoma remains poor and approximately 70% of patients present with recurrent disease within a year. Stupp et al. reported a 14.6% median OS rate and only 26.5% of treated patients in their study were alive at the 24-month follow-up evaluation, but PFS at 6.9 months was 53.9%.[22] Nonetheless, the above data, albeit derived from a TMZ chemotherapeutic treatment performed up

to 6 cycles only, clearly indicate a clinical benefit from the association of TMZ chemotherapy and radiotherapy versus radiotherapy only or other chemotherapeutic regimens previously used.

Long-Term TMZ Therapy Versus a Standard Protocol

A literature review on long-term administration of TMZ in newly diagnosed glioblastomas revealed scant data, with only 4 studies addressing such a topic.[6,8,10,16] Khasraw et al. treated 3 patients with TMZ for 5, 7, and 8 years, respectively, but only 1 with a glioblastoma. Interestingly, 2 of their patients showed tumor recurrence following interruption of TMZ and responded well to reintroduction of TMZ.[8]

Mannas et al. reported their results in 5 patients treated with long-term TMZ, i.e., from 45 to 85 cycles. The only patient with glioblastoma, although treated with concomitant and adjuvant TMZ at a standard dose, developed a recurrence 2 years and 10 months after resection. His dosage was then increased to 75 mg/m^2 for 14 days per a 28-day cycle and he did not present with any hematological toxicity. Following MRI evidence of partial tumor reduction, the TMZ dose was returned to 150 mg/m^2 for 5 days of a 28-day cycle. However, it is important to highlight that when the patient decided to stop the TMZ therapy because of a minor thrombocytopenia, he died 6 weeks later, having undergone 56 cycles of TMZ.[10] Such experience is similar to ours: 2 of our patients in Group B died 6 weeks after stopping TMZ.

In 2007, Hau et al. published data collected from 50 German centers on the use of TMZ for at least 12 cycles or 12 months in patients with glioblastoma.[6] In those patients, TMZ administration was stopped because of toxicity or evidence of recurrent disease. Seventy-three patients with primary glioblastoma and 65 subjects with recurrent disease received a median of 13 TMZ cycles (range 9–40 cycles) and 14 cycles (range 11–40 cycles), respectively. No signs of tumor progression were observed during therapy in either group. In this analysis, PFS was 14 months (range 10–40 months) for patients treated with first-line TMZ, and median OS was 30.6 months for patients with glioblastoma and 22.4 months from initiation of TMZ. The longest survival time was 58.5 months in 1 patient with glioblastoma; another patient with recurrent glioblastoma in their series survived 66 months (time to progression after first-line TMZ was 8.3 months). Hau et
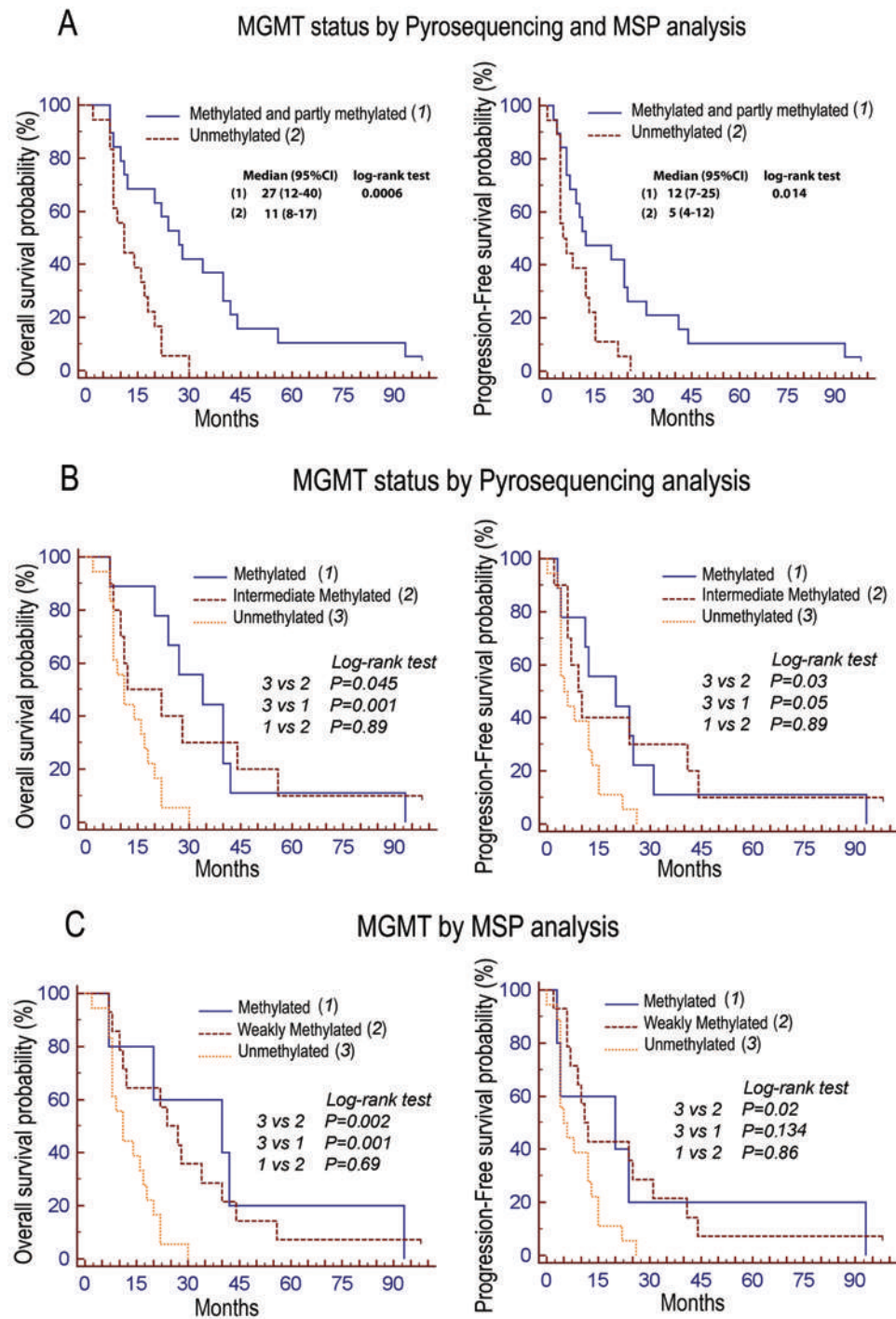
FIG. 3. Comparison of OS and PFS according to MGMT methylation status by Kaplan-Meier curve analysis. **A:** Overall survival and PFS in patients with glioblastomas are correlated with MGMT promoter status in 2 patient groups (methylated and unmethylated), which are stratified by pyrosequencing and MSP analysis methods. **B and C:** Overall survival and PFS are compared with MGMT status of the 3 patient groups (methylated, intermediate/weakly methylated, and unmethylated), ordered according to methylation degree identified by pyrosequencing analysis (B) and MSP analysis (C).

al. reported an overall 2-year survival time of 68% (50 patients) in the primary glioblastoma group of patients treated with long-term TMZ and concluded that long-term TMZ therapy is feasible and well tolerated.[6]

Seiz et al. found a statistically significant correlation between PFS and OS and the number of TMZ cycles in

59 of 114 patients treated with long-term TMZ therapy (range 6–57 cycles).[16] Their patients' median survival and 2-year survival were 15 months and 27.5%, respectively. In the study of Seiz et al., TMZ therapy was stopped because of toxicity (34%), tumor progression, patient's wish (4%), or unspecified reasons (39%).
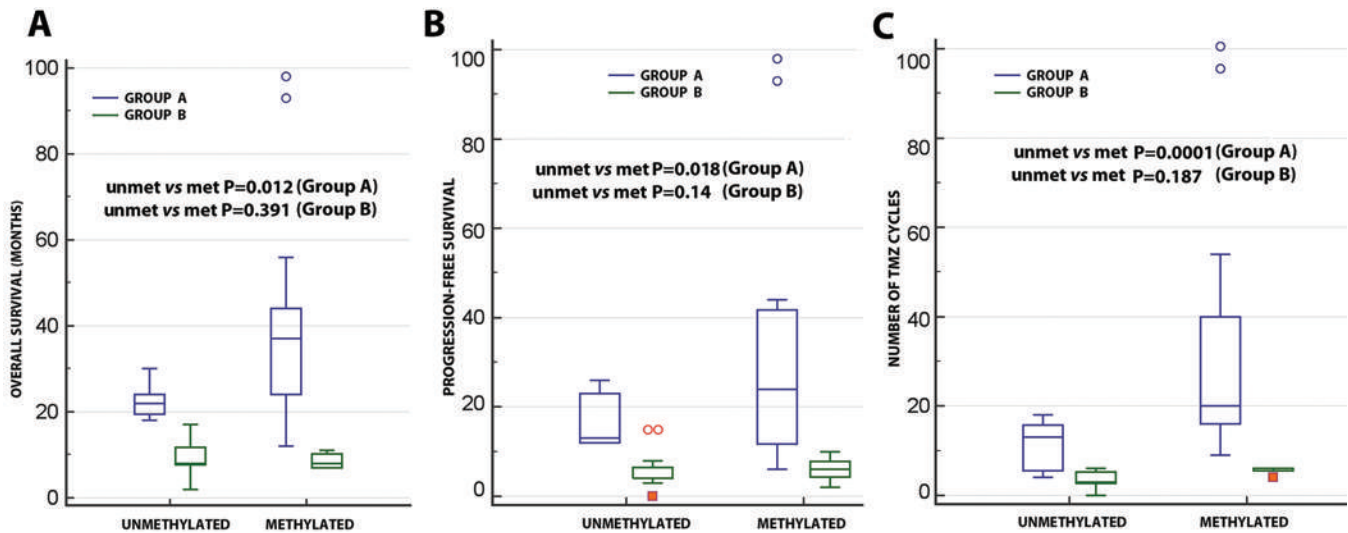
Fig. 4. Boxplot representation of OS **(A)**, PFS **(B)**, and number of TMZ cycles **(C)** by MGMT methylation status in Group A and B patients. The *central box* represents the values from the lower to upper quartile (25th–75th percentile). The *middle line* represents the median. The *horizontal line* extends from the minimum to the maximum value, excluding outside and far out values (shown as *circles* and *squares*).
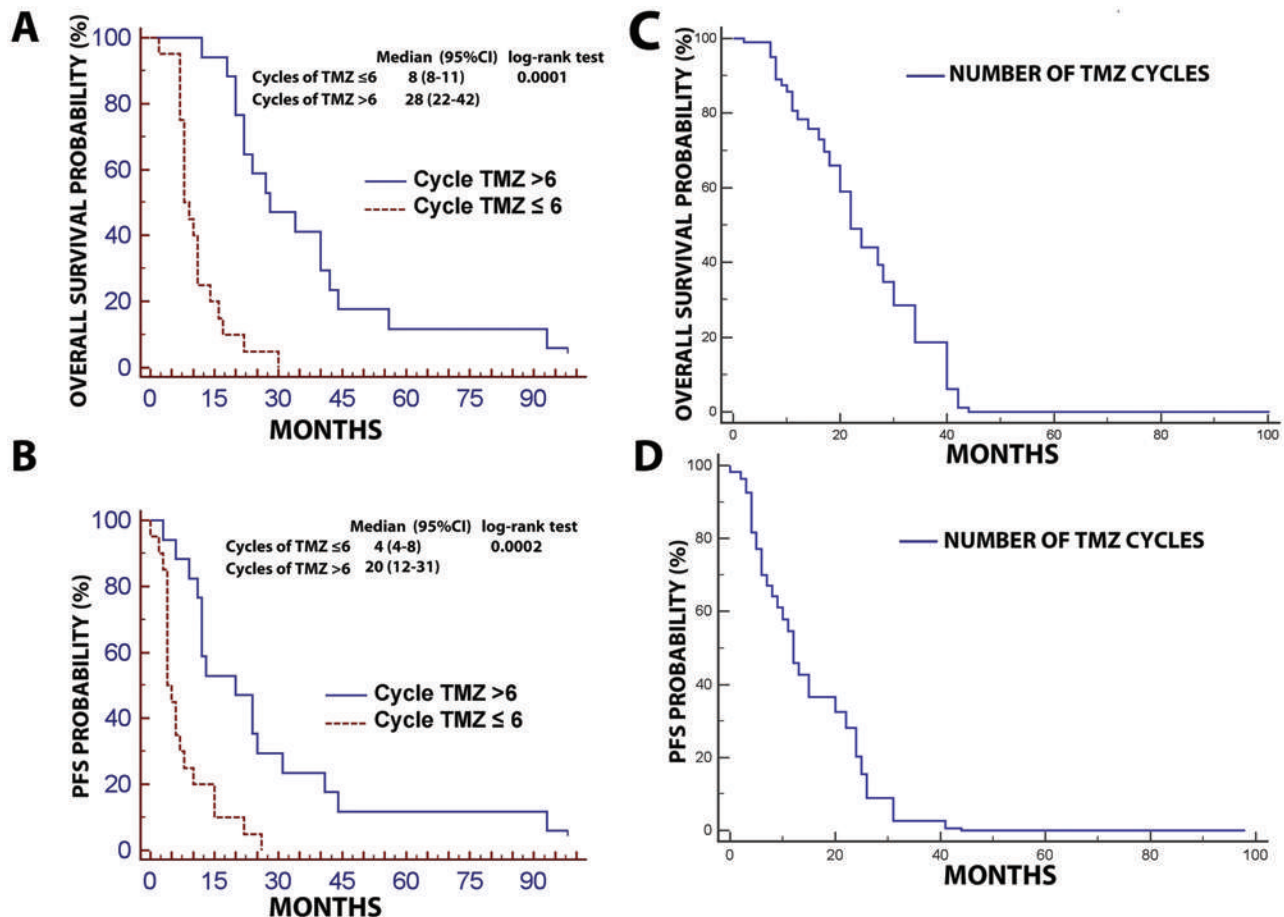

Fig. 5. Kaplan-Meier analysis correlating OS and PFS of Group A and B patients to number of TMZ cycles **(A and B)**, and correlating OS and PFS of patients with glioblastoma to number of TMZ cycles **(C and D)**.

Our data on PFS and OS suggest that the median OS in our series is 28 months (range 22–42 months) in patients treated with more than 6 TMZ cycles compared with 8 months (range 8–11 months) in patients treated with no more than 6 cycles, and such a difference is highly statistically significant (p = 0.0001). Median PFS was also different between the 2 groups, i.e., 20 months (range 12–31 months) in those undergoing more than 6 TMZ cycles compared with 4 months (range 4–8 months) in patients treated with no more than 6 cycles (p = 0.0002; Fig. 5).

Both our data and those reported by Hau et al.[6] and by Seiz et al.[16] demonstrate a correlation between the number of TMZ cycles and OS and PFS. In all 3 studies, patients treated with long-term TMZ (> 6 cycles) had a longer PFS and OS. Comparing our median OS for patients treated with more than 6 TMZ cycles with those reported in the other 2 published series,[6,16] it is important to observe that our data are consistent with the median survival of the patients in the Hau et al. study, who survived 30.6 months.[6] Seiz et al. reported a 15-month median survival, but if their data included the patients who were still alive (which had been censored in the statistical analysis), median survival would have been 28.5 months.[16]

Other experiences with long-term TMZ treatment have been reported in patients with recurrent high-grade glioma. Colman et al. found a statistically significant difference in PFS between patients treated with 12–18 cycles compared with patients receiving 19 cycles or more of TMZ,[2] and such findings are consistent with our results in newly diagnosed glioblastomas. Yet it remains unclear what the impact of the early TMZ cycle at a dose of 150 mg/m^2 is on OS and PFS, which we administered to all our patients soon after surgery while still on the neurosurgical ward. This is, however, a clear difference between both the standard Stupp protocol and the studies of Hau et al. and Seiz et al., and we submit that its potential value deserves further investigation.

Role of MGMT Promoter Methylation Status

The survival benefit of MGMT gene silencing by promoter methylation in patients with glioblastoma undergoing TMZ chemotherapy and radiotherapy has been demonstrated by Hegi et al. These investigators reported a 21.7-month median survival in patients with methylated MGMT promoter who were treated with radiotherapy and TMZ, compared with 15.3 months in patients treated with radiotherapy only,[7] and MGMT methylation analysis is now widely used.[3,13,17,24,26] We analyzed our patients' survival in relation to MGMT promoter methylation status and found a statistically significant correlation: median survival in patients with methylated or partially methylated MGMT was 27 months (95% CI 12–40 months) versus 11 months (95% CI 8–17 months) in patients with unmethylated MGMT (p = 0.0006, log-rank test). Median PFS was 12 months (95% CI 7–25 months) in patients with methylated or partially methylated MGMT and 5 months (95% CI 4–12 months) in patients with unmethylated MGMT (p = 0.014, log-rank test; Fig. 3).

Interestingly, our findings suggest that the extent of methylation impacts survival. Nonetheless, because of the limited data for each of the 3 patient subsets (average methylation < 9%, 9%–25%, and > 25%), we did not use methylation extension as a factor to build a prognostic model. Further studies on larger cohorts of patients are needed to address this issue.

Extent of Tumor Resection

In addition to chemoradiotherapy, the impact of tumor EOR has been clearly demonstrated. Sanai and Berger[14] and Sanai et al.[15] proved that in newly diagnosed glioblastomas an EOR above 78% correlates with significant survival advantage. Such benefit further improves when EOR is between 95% and 100%, as documented by postoperative MRI. Yet the use of 5-ALA and fluorescence-guided resection may help to increase tumor EOR and achieve a better outcome.[21] Published studies on long-term TMZ therapy report different data when EOR is considered. One of the most important biases in the study of Hau et al. is the tumor EOR, which was reported to be total in only 50% of their patients. As a consequence, although Hau et al. highlighted the tolerability of long-term TMZ and stressed the low incidence of toxicity, their results in terms of both PFS and OS might have been different (i.e., even better) if a higher percentage of patients were treated with tumor GTR. Yet Seiz et al. found a statistically significant correlation between PFS and OS and the number of TMZ cycles in 59 of 114 patients treated with long-term TMZ therapy (range 6–57 cycles), but were unable to link the chemotherapy regimen to tumor EOR. Only 51 of their 114 patients underwent GTR.[16]

Conversely, all but 2 of our patients (1 each in Group A and Group B who had STRs) underwent tumor GTR as defined by Sanai et al.[15] and confirmed by early postoperative volumetric MRI scans. We believe that analysis of long-term TMZ efficacy could produce different (i.e., better) results with regard to PFS and OS according to the tumor EOR and the latter should not be overlooked. We submit that tumor EOR is one of the most important differences between our study and the studies of Seiz et al.[16] and Hau et al.[6]

With regard to the role of 5-ALA in EOR, we could not find any difference in this study among patients treated without (n = 22) or with 5-ALA (n = 15), as independent and blinded radiological evaluation of early postoperative MR images confirmed GTR in all but 2 patients, regardless of the use of 5-ALA, involving 1 recurrence in 1 patient initially treated without 5-ALA and 1 in a case operated on using 5-ALA.

Toxicity Associated With TMZ Therapy

Temozolomide is a better tolerated drug than other chemotherapeutic agents, with few reported complications such as opportunistic infections, or hematological toxic effects such as neutropenia or pancytopenia, presenting in about 8% of patients at variable time intervals.[19,22] However, the potential risk of cumulative toxicity has likely halted the standardized spread of long-term therapy with TMZ.

Hau et al. noted that the overall incidence of toxicity was very low in their study and consistent with data reported by Stupp et al., and described only 10% cases of throm-

bocytopenia, 7% of leukopenia, 5% of gastrointestinal toxicity, and 4% of infection.[6] However, these authors did not specify whether the above complications occurred in the cohort of primary glioblastomas or in the recurrent ones, which they also included in their study. Seiz et al. reported that 39 patients in their study (34%) had treatment stopped because of hematological side effects (such as thrombocytopenia and pancytopenia) but only 17% of those were included in the long-term TMZ group (> 6 cycles).[16]

In our Group A patients (those treated with > 6 cycles of TMZ), treatment was interrupted because of either disease progression or the patient's wish (n = 1), but severe toxicity was never the cause. Our data suggest a limited incidence of nausea and vomiting. As opposed to previous studies,[6,16,22] no hematological toxicity was diagnosed in our patients despite prolonged treatment with as many as 101 TMZ cycles, but only minor and transient reduction in platelets and/or white blood cell counts. Such changes, however, did not prompt TMZ therapy interruption. We are aware that it is difficult to explain these findings, and the lower daily dose of TMZ we used, even during adjuvant cycles (150 mg/m^2 rather than 200 mg/m^2 as in other studies), could be only a possible explanation.

Interestingly, such a hypothesis is also supported by the experience of Mannas et al. In 1 of their patients they used a different TMZ protocol and administered a lower dose (75 mg/m^2), albeit for a longer time (14 days per 28-day cycle), and did not observe any toxicity.[10]

Predictors of Response to Treatment and Survival

Several studies have tried to identify statistically significant factors such as age and functional status that predict outcome in patients with glioblastoma.[14] Reviewing the 4 published studies on long-term TMZ therapy,[6,8,10,16] we found that only Seiz et al. reported that age had a role in their treatment paradigm, with those patients receiving more than 6 TMZ cycles younger than those receiving no more than 6 cycles, and the number of TMZ cycles did have a statistically significant correlation with improved PFS and OS in their study.[16] In the present study, age and KPS score were shown by univariate Cox proportional hazard regression analysis to be prognostic factors and to correlate with OS and PFS ($p < 0.05$). However, in a multivariate Cox proportional hazard regression analysis, a statistically significant correlation was not found for age and KPS score in relation to OS and PFS (Table 3). Moreover, we observed that younger patients (≤ 60 years old) lived longer than older ones (> 60 years old), both in the group with longer TMZ intake as well as in the group with shorter TMZ intake (Table 2). The growth fraction, as determined by the antibody Ki 67, shows regional heterogeneity within a glioblastoma. Despite the wide range of proliferation indices observed in glioblastoma, an association between Ki 67 proliferation index and clinical outcome has not been demonstrated.[12]

Conclusions

Our study has some unique features: 1) it describes the longest experience so far reported with TMZ in patients with newly diagnosed glioblastoma (up to 101 cycles), who were treated using MRI-proven surgical GTR; 2) patients were started early on TMZ treatment, usually on the second or third postoperative day, while still on the neurosurgical ward; 3) the usual dose for adjuvant TMZ cycles was 150 mg/m^2; 4) no significant hematological toxicity was encountered among patients treated with long-term TMZ; and 5) a statistically significant correlation was found between median survival and number of TMZ cycles, as well as between median survival and MGMT promoter methylation status. The retrospective nature of our study and the small number of patients are limitations of the study. Nonetheless, our statistically significant data, together with the other few reported experiences in the literature as well as the unreported common clinical experiences to continue TMZ therapy if well tolerated by patients, should encourage the design of a prospective, randomized, multicenter study on the efficacy and safety of prolonged TMZ chemotherapy in patients with newly diagnosed glioblastoma treated with proven tumor GTR.

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