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O^6-methylguanine DNA-methyltransferase methylation status can change between first surgery for newly diagnosed glioblastoma and second surgery for recurrence: clinical implications

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O^6-methylguanine DNA-methyltransferase (*MGMT*) promoter methylation status is a prognostic factor in newly diagnosed glioblastoma patients. However, it is not yet clear whether, and if so how, *MGMT* methylation status may change. Moreover, it is unknown whether the prognostic role of this epigenetic feature is retained during the disease course. A retrospective analysis was made using a database of 614 glioblastoma patients treated prospectively from January 2000 to August 2008. We evaluated only patients who met the following inclusion criteria: age ≥18 years; performance status 0-2; histological diagnosis of glioblastoma at both first and second surgery for recurrence; postoperative treatment consisting of: (i) radiotherapy (RT) followed by adjuvant temozolomide (TMZ) until 2005 and (ii) TMZ concurrent with and adjuvant to RT after 2005; a time interval ≥3 months between first and second surgery. *MGMT* status was evaluated at first and second surgery in all 44 patients (M:F 32:12, median age: 49 years, range: 27–67 years). In 38 patients (86.4%), *MGMT* promoter status was assessable at both first and second surgery. *MGMT* methylation status, changed in 14 patients (37%) of second surgery samples and more frequently in methylated than in unmethylated patients (61.5% vs 24%, $P = .03$). The median survival was significantly influenced only by *MGMT* methylation status determined at first surgery ($P = .04$). Significant changes in *MGMT* methylation status during the course of GBM occur more frequently in *MGMT* methylated than unmethylated cases. *MGMT* methylation status determined at first surgery appears to be of prognostic value; however, it is not predictive of outcome following second surgery.

Keywords: glioblastoma, second surgery, temozolomide, *MGMT*, recurrence

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Data recently reported in the randomized EORTC 22981/26981–NCIC CE.3 (EORTC/NCIC) phase III trial[1] on subjects given temozolomide

(TMZ) concurrent with and adjuvant to radiotherapy (RT) have provided a new standard of care for newly diagnosed GBM patients. Moreover, in the companion study by Hegi et al.[2], O^6-methylguanine DNA-methyltransferase (*MGMT*) gene promoter methylation status was found to be a potent prognostic factor in these patients. The *MGMT* gene encodes for a DNA repair protein involved in TMZ DNA damage removal, and the epigenetic silencing by the methylation of its promoter is correlated with the cytotoxic effect of TMZ on methylating the O6 position of guanine.

However, there are still many open questions concerning the role of this prognostic factor, and it has yet to be established, in particular, whether *MGMT* methylation status assessed during the course of the disease, rather than at the diagnosis, as done in the EORTC/NCIC trial, has an equally valuable role in predicting clinical outcome.

In the present study, an evaluation was therefore made of *MGMT* methylation status in patients who underwent at least 2 surgical procedures and, during the time interval between the 2 operations, received RT followed by adjuvant TMZ or TMZ concurrent with and adjuvant to RT (RT/TMZ).

Methods

Patient Eligibility

A retrospective analysis was made of a database of 614 GBM patients followed prospectively between January 2000 and August 2008. We evaluated only patients who met the following inclusion criteria: age $\geq$18 years; performance status (PS) at diagnosis 0-2; histological diagnosis of GBM both at first and at second surgery for recurrence; postoperative treatment consisting of: (i) RT followed by adjuvant TMZ until 2005 and (ii) TMZ concurrent with and adjuvant to RT after 2005;[1] a time interval of $\geq$3 months between first and second surgery. Determination of *MGMT* promoter methylation status in tumor tissue samples obtained at both surgical procedures was mandatory. The extent of surgical excision was determined on the basis of the surgeon's estimation reports. All patients signed a form giving their fully informed consent to participate in the study by undergoing treatment and translational research; the study protocol was approved by the Institutional Review Boards of Padova and conducted according to the principles of the Declaration of Helsinki and the rules of Good Clinical Practice.

Treatment Plan

Radiotherapy. RT consisted of a conventionally fractionated regimen, with the delivery of a total dose of 60 Gy in 6 weeks, in a once daily schedule of 2 Gy per fraction for a total of 30 fractions. The gross tumor volume consisted of the entire visible tumor at preoperative contrast enhanced CT or MRI. The clinical target volume included the entire enhanced tumor (according to preoperative contrast CT or MRI) plus a 2–3 cm margin.

Chemotherapy

The postsurgical treatments allowed were: (i) RT followed by adjuvant TMZ (150–200 mg/m^2 for 5 days every 28 days) before 2005 and (ii) RT plus continuous daily TMZ (75 mg/m^2/day), followed by adjuvant TMZ (150–200 mg/m^2 for 5 days every 28 days) as described elsewhere, after 2005.[3] In both cases, TMZ treatment was suspended after 12 cycles only if the MRI showed no enhancement suggesting tumor presence; otherwise chemotherapy was delivered until complete response or clear disease progression. Patients were evaluated every 2 cycles taking into account the results of radiological, clinical, and neurological examinations according to the Macdonald's criteria. Neurological status was assessed by considering signs and symptoms that, with respect to the previous examination, were possibly correlated with progression; any variation in daily corticosteroids dosage was recorded.

MGMT Status Assessment

MGMT status was evaluated by means of the methylation-specific polymerase chain reaction following a nested-polymerase chain reaction protocol,[4] using methods and assessment criteria described elsewhere,[5] on both the surgical specimens for all the patients. Tissue blocks were selected for DNA extraction after careful examination on hematoxylin and eosin staining of corresponding sections to rule out the presence of contaminating necrotic debris. Molecular genetic analyses were performed on samples with an estimated tumor cell content of at least 90% from 5 sections of 10 μm from paraffin-embedded tissue blocks. The tumor area was either macrodissected manually using a sterile blade or microdissected using the laser-assisted SL μcut Microtest (MMI GmbH distributed by Nikon, Firenze, Italy, http://www.mmi-micro.com). Four 10-minute incubations, 2 with xylene at 60°C and 2 with absolute ethanol at room temperature, were used to eliminate paraffin. The tissues were then lysed with proteinase K at 55°C overnight. Genomic DNA was extracted using the GENTRA Puregene tissue kit (Qiagen, Milan, Italy) in accordance with the manufacturer's instructions. The pellet was then eluted in 35 μL of TE buffer, and total DNA was quantified by Quant-iT^{TM} dsDNA BR kit (Invitrogen, Carlsbad, CA). At least 200 ng of DNA was then treated with bisulphite using the EpiTect Bisulphite kit (Qiagen) according to the manufacturer's instructions.

To test for the sensitivity and specificity of nested MS-PCR, titration experiments were performed using normal pooled genomic DNA (DNA female pool, Cod. G1521, Promega), which was methylated *in vitro* using *SssI* (New England Biolabs, Ipswich, MA). In brief, 1.5 μg of DNA was treated with *Sss*I to methylate all CpG sites (near complete methylation and no loss of

DNA was assumed) for 2 hours at 37°C following the supplier's instructions. Mixtures of *Sss*I-treated DNA and untreated DNA (100%, 50%, 10%, 1%, 0.1%, and 0.01%), were prepared in duplicate (each containing 1.5 μg of template DNA). Nested MS-PCR was performed as described previously by Palmisano et al.,[4] with minor modifications: a total of 26 cycles for the flanking primers and a total of 30 cycles for the methylation-specific primers were performed. Amplicons were detected by SeaKem LE agarose gel (3%, Lonza, Milan, Italy) by using GelStar (Lonza) as the intercalator. The results obtained were verified using a second step for the nested polymerase chain reaction; in some cases, the entire process was repeated in triplicate. The analytical sensitivity limit of the nested MS-PCR assay was determined using serial dilution mixtures of *Sss*I-treated DNA and untreated DNA (100%, 50%, 10%, 1%, 0.1%, and 0.01% each containing 1.5 μg of template DNA). On using this protocol, a sensitivity of 0.01% was detected for methylated alleles and confirmed in different runs. The specificity of the assay was assessed by purification and analysis of a series of DNA from the whole blood of 12 healthy donors in which no methylated allele was detected.

Statistical Analysis

Tumor progression was defined, according to the Macdonald's criteria,[6] as a 25% increase in tumor size, the appearance of new lesions, or an increased need for corticosteroids. Time to progression and survival, measured as from the time of first surgery to disease progression or death, respectively, or date of last follow-up, were analyzed using the Kaplan–Meier method; 95% confidence intervals (CI) were calculated using the associated estimated standard errors. The log-rank test was employed to test the significance of the following prognostic variables: *MGMT* promoter methylation status, age, gender, extent of surgery, performance status, and pattern of disease recurrence. The percentage of *MGMT* methylated at first and second surgery was compared using the McNemar test. Significance was set at $P < .05$.

Results

Forty-four patients (M:F 32:12, median age: 49 years, range 27–67 years) met the inclusion criteria. The study patients' baseline characteristics are listed in Table 1. The median time interval between the first and second surgery was 12 (range 4–48) months.

MGMT Methylation Status

MGMT status, determined in all 44 patients both at first surgery and at the time of second surgery, was assessable in 40 (90.9%) patients at first surgery and in 42 (95.5%) patients at second surgery. Results from both specimens were assessable in a total of 38 (86.4%) patients. *MGMT* promoter was methylated in 13 cases (34.2%) at first surgery and in 11 cases (28.9%) at second surgery (McNemar test, $P = 0.5$). At the evaluation of second surgery samples, *MGMT* methylation status was unchanged in 24 patients (63.2%); of these, 5 (24%) had *MGMT* methylated and the other 19 (76%) had *MGMT* unmethylated status. In 8 of 13 (61.5%) cases, *MGMT* status changed from methylated to unmethylated, and in 6 of 25 (24%), a conversion occurred from *MGMT* unmethylated to methylated (Fig. 1). The change in *MGMT* status between first and second surgery was more frequent in patients with *MGMT* methylated promoter status than in patients with *MGMT* unmethylated promoter status (2-tailed Fisher's exact test, $P = .03$).

Table 1. Patients' characteristics

		Population with *MGMT* evaluable in both specimens ($n = 38$)
Gender (M:F)		28:10
Age (y) at diagnosis		49 (range 27–67)
Surgery	Biopsy	1 (2.6%)
	Partial resection	10 (26.3%)
	Complete resection	27 (71.1%)
Treatment after initial surgery	RT and sequential TMZ	11 (28.9%)
	RT/TMZ	27 (71.1%)
Time between the 2 resections (mo)		12 (range: 4–48)
MGMT methylation status at first surgery	Methylated	13 (34.2%)
	Unmethylated	25 (65.8%)
MGMT methylation status at second surgery	Methylated	11 (28.9%)
	Unmethylated	27 (71.1%)

MGMT, O^6-methylguanine DNA-methyltransferase; RT, radiotherapy; TMZ, temozolomide.

Influence of Treatment on MGMT Status

Among the 38 patients with evaluable *MGMT* results from samples collected at both first and second surgery, 11 (29%) received RT followed by TMZ and 27 (71%), concurrent RT/TMZ treatment. TMZ was not administered concurrently with RT for several reasons: treatment given before EORTC/NCIC data[1] were available (9 patients); non-GBM diagnosis subsequently redefined as GBM (1 patient); or preexisting comorbidities (1 patient).

In 13 patients with *MGMT* methylated in the surgical specimen obtained at the first surgery, the type of treatment given after first surgery significantly affected the pattern of *MGMT* status variation at recurrence. *MGMT* methylation status was constant in 1 of 8

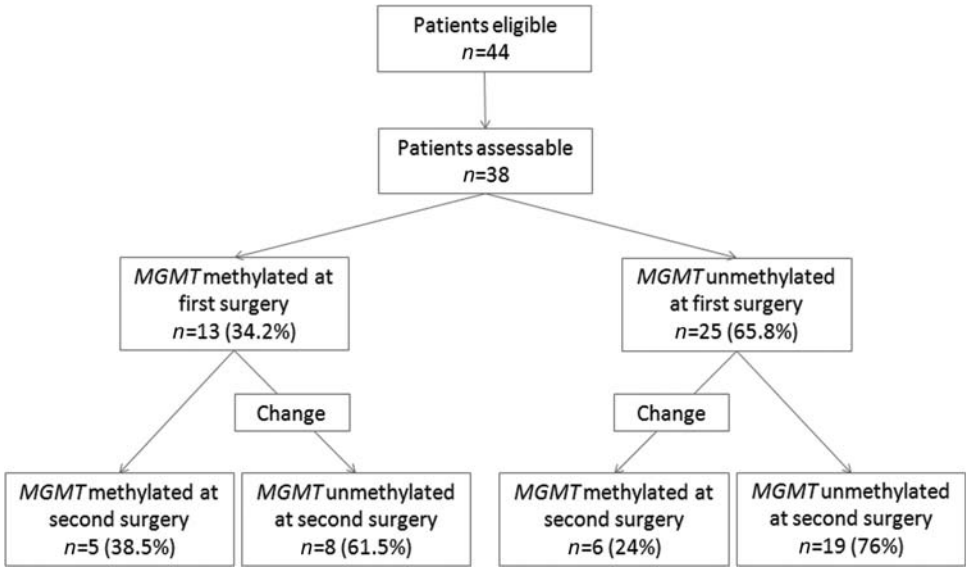

Fig. 1. *MGMT* methylation status at first and second surgery. Changes in *MGMT* methylation status occurred more frequently in patients with an initially methylated *MGMT* status (2-tailed Fisher's exact test, $P = .03$).

(12.5%) patients treated with concurrent RT/TMZ and 4 of 5 (80%) patients given RT with sequential TMZ ($P = .03$). On the other hand, in the 25 patients with *MGMT* unmethylated status at first surgery, the pattern remained unchanged irrespective of treatment given.

Overall Survival

The median survival was 24.3 months (95% CI 20.8–27.7) and 35.2 months (95% CI 10.1–60.3) and 21.9 months (95% CI 17.3–26.5) for *MGMT* methylated and unmethylated patients at the time of first surgery, respectively (log-rank, $P = .04$; Fig. 2). The extent of surgical resection was not correlated with survival ($P = .5$), nor was type of postsurgical treatment ($P = .2$); however, *MGMT* methylation status was not well balanced in these treatment groups, being 5 of 11 (45.5%) and 8 of 27 (29.6%) for the *MGMT* methylated patients in the RT group and RT/TMZ group, respectively. The median survival time interval between the times of first and second surgeries was 9.7 months (95% CI 8.6–10.8) and 22.1 months (95% CI 8.0–36.2) in patients with *MGMT* methylated status at the time of first surgery and 9.7 months (95% CI 6.4–13.0) in patients with *MGMT* unmethylated status at the time of first surgery ($P = .13$). However, *MGMT* status at the time of second surgery was prognostic neither for survival ($P = .1$; Fig. 3) nor for survival from the time of second surgery ($P = .7$; Fig. 4). Moreover, survival following second surgery was not significantly correlated with any of the variables analyzed.

After second surgery, 33 patients received further chemotherapy, consisting of TMZ rechallenge in 25 patients, nitrosoureas-based chemotherapy in 7 patients, and bevacizumab–irinotecan in 1 patient. The type of second-line chemotherapy administered after second surgery to patients with methylated or unmethylated tumors did not differ significantly and was therefore comparable, being administered to 9 of 11 (81.8%) *MGMT* methylated patients and 24 of 27 (88.9%) *MGMT* unmethylated patients.

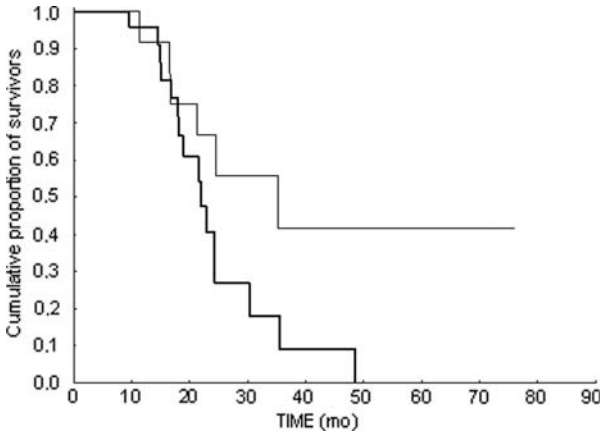

Fig. 2. Survival according to *MGMT* methylation status determined at first surgery. Thin line, patients with methylated *MGMT* promoter; bold line, patients with unmethylated *MGMT* promoter status.

Discussion

MGMT methylation status at the time of first surgery has been shown to be a potent prognostic factor for patients treated with either RT followed by TMZ or TMZ concurrent with and adjuvant to RT.[2] However, it is unclear whether this epigenetic feature is consistent also at the time of disease recurrence after postsurgical RT followed by TMZ and whether its prognostic role

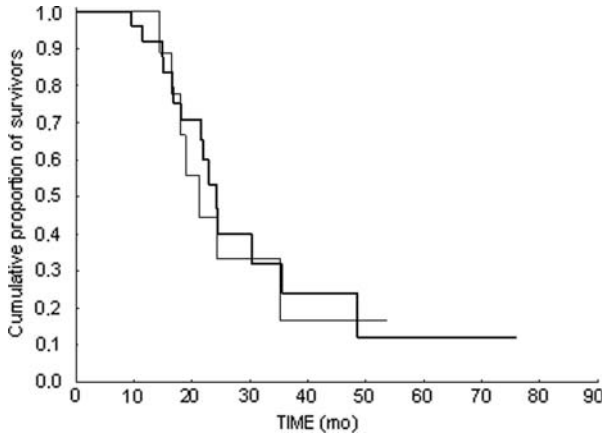

Fig. 3. Survival according to *MGMT* methylation status determined at second surgery. Thin line, patients with methylated *MGMT* promoter; bold line, patients with unmethylated *MGMT* promoter status.

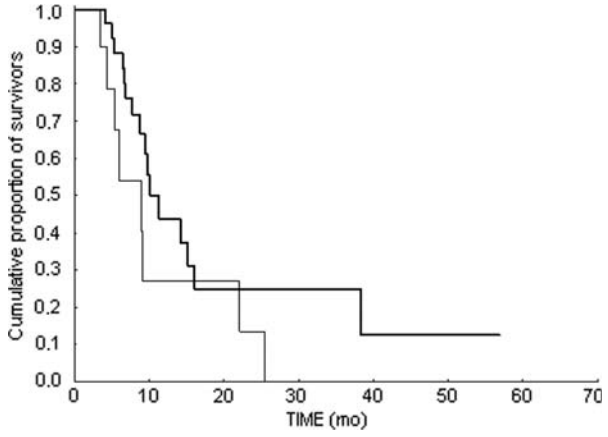

Fig. 4. Survival after second surgery according to the *MGMT* methylation status determined at second surgery. Thin line, patients with methylated *MGMT* promoter; bold line, patients with unmethylated *MGMT* promoter status.

is retained. In a consecutive and prospectively recorded database of GBM cases, we therefore evaluated data from tumor specimens obtained during first and second surgery. *MGMT* methylation status was determined in both specimens, and all patients who underwent second surgery had undergone RT followed by TMZ or concurrent RT/TMZ in the time interval between first and second surgery.

Concordance between *MGMT* methylation status at the time of first surgery and second surgery was relatively low (63%). Moreover, we found that *MGMT* status changed more frequently in patients with *MGMT* methylated (61.5%) than in patients with *MGMT* unmethylated (24%) status at first surgery ($P = .03$). To our knowledge, this is the largest series in which pair-wise testing for MGMT at diagnosis and recurrence of GBM has been investigated. Our observations are based on an experience of only 14 patients with *MGMT* changes in methylation status. However, the results reported in the present study let us speculate that the type of postsurgical treatment may influence *MGMT* methylation status variation: patients treated with concurrent chemotherapy-RT were characterized by a substantially high percentage of *MGMT* shifts from *MGMT* methylated status at the time of first surgery to unmethylated status at the time of second surgery ($P = .03$). Yet, owing to the small cohort of patients in this subgroup, no definitive conclusions can be drawn. Interesting findings regarding this issue have been reported by The Cancer Genome Atlas Research Network on treated GBMs, in which *MGMT* methylation status together with a mismatch repair system, in the context of treatments received, have been shown to significantly influence the pattern and frequency of somatic point mutations[7] because of a hypermutator phenotype following the lack of DNA repair systems.

Nevertheless, changes in *MGMT* methylation status between primary disease and recurrence have raised the issue of GBM progression after highly aggressive treatment and after the sometimes long-standing latency of microscopical residual disease. *MGMT* methylation pattern heterogeneity within the tumoral tissue could be taken into account, despite the apparently contradictory data in the literature.[8,9] In fact although some data seem to confirm homogeneity in *MGMT* methylation patterns in the whole tumor burden, other data seem to suggest variation in the methylation status of the MGMT promoter after treatment or across different regions of the same tumor. However, minimal residual disease consisting of chemoresistant cells might be responsible for recurrence. Cancer stem cells (CSC), which may account for minimal residual disease, have properties enabling the generation of new tumors. CD133+ and CD133− CSC have been found to coexist in glioma-initiating populations.[10] Interestingly, CD133+ CSC are characterized by a lower level of MGMT RNA and protein expression.[11,12] Thus, an effective treatment, such as RT/TMZ, could deplete the population expressing MGMT and comprising CD133− cells, whereas CD133+ cells with lower MGMT levels would be resistant to treatment and generate recurrence in response to unknown stimuli.

Intriguingly, findings from in vitro studies show that TMZ does not induce cell death but efficiently inhibits proliferation in CD133+ CSC lines,[12] showing a cytostatic activity rather than cytotoxic, thus explaining the long time periods during which there is no clinical evidence of recurrence.

Another interesting finding made in the present study was that overall survival and survival time after second surgery were not correlated with concurrently determined *MGMT* methylation status, despite the fact that the majority of patients were treated with alkylating agents after second surgery. These findings should be confirmed in a larger number of unselected patients.

Moreover, as previously shown by Sadones et al.,[13] also in our analysis *MGMT* methylation status evaluated at the time of first surgery was not predictive of survival from the time of tumor recurrence. This may have

depended in part on the low activity of second-line therapies, especially those with alkylating agents, at the time of failure after RT/TMZ treatment. Moreover, the alterations in other mechanisms implicated in TMZ sensitivity may explain the lack of predictivity of *MGMT* methylation status at recurrence. Overall, although in the population selected by us, prognostic factors were favorable (ie median age, 49 years, and debulking surgery obtained in 97% of patients), our findings suggest that the *MGMT* methylation pattern, and its prognostic value, may change during the course of GBM. It is hoped that future studies will address the biology of this disease in relation to treatments and evaluate CSC as a potential novel target approach.

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Conflict of interest statement. A.A.B. is a member of the advisory boards of Schering-Plough, Bristol-Meier Squibb, Roche, and Oncomethylome Sciences and a speaker for Schering-Plough, Glaxo, and Roche.

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