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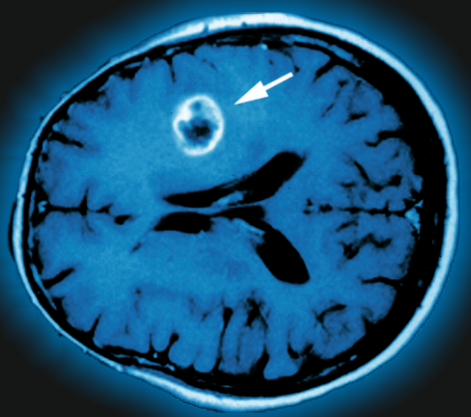

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Original Article

O-6-methylguanine-DNA methyltransferase promoter methylation can change in glioblastoma recurrence due to intratumor heterogeneity

Valeria Barresi[1], Maria Caffo[2], Giuseppa De Luca[3], Giuseppe Giuffrè[4]

[1]Department of Diagnostics and Public Health, University of Verona, Verona, Italy.
[2]Department of Biomedical Sciences and Morphological and Functional Images, University of Messina, Messina, Italy.
[3]Ircss Policlinico San Martino, Genova, Italy.
[4]Department of Human Pathology in Adulthood and Evolutive Age, University of Messina, Messina, Italy.

Abstract

Background and Aim: The standard-of-care for patients with glioblastoma (GBM) is surgery followed by concurrent chemotherapy with temozolomide and radiotherapy. O-6-methylguanine-DNA methyltransferase (*MGMT*) promoter methylation is commonly assessed in GBM as a predictive marker of response to temozolomide. Although *MGMT* methylation status has been shown to change between primary and recurrent GBM, no indication exists on retesting MGMT in recurrent GBM. In addition, what causes the change in *MGMT* methylation has yet to be identified. In this study, we aimed to investigate whether MGMT promoter methylation in recurrent GBM was influenced by intratumor heterogeneity in the initial GBM tumor. **Materials and Methods:** We investigated the status of *MGMT* promoter methylation in different samples taken from concentric layers of 24 GBMs and in 11-paired surgically resected recurrences. The neoplastic nature of samples submitted for methylation analysis was preliminary verified through histological examination; the fragments were accurately chosen to have adequate cellularity and minimal amount of nontumor contaminants. **Results:** About 27% (3 out of 11) of the recurrences had changed *MGMT* methylation status compared to the initial tumor. Initial tumor heterogeneity might play a role in this change, as all three cases had intratumor heterogeneity (with the central part of the tumor methylated and the peripheral part unmethylated) in the primary GBM. **Conclusion:** This study suggests that *MGMT* methylation variation in recurrent GBM may depend on intratumor heterogeneity in the initial tumor. Intratumor heterogeneity and possible changes in the recurrence should be taken into account when testing *MGMT* promoter methylation status as a predictive factor orienting therapeutic decisions in patients with GBM.

Keywords: Glioblastoma, heterogeneity, IDH, O-6-methylguanine-DNA methyltransferase, recurrence

Introduction

Glioblastoma (GBM) is one of the most frequent primary tumors of the central nervous system (CNS).[1] According to the World Health Organization classification of CNS tumors, it can be subdivided into *IDH* mutant and *IDH* wild-type, on the basis of the mutational status of *IDH1/IDH2* genes.[1] GBM *IDH* wild-type is the most common subtype; it mainly affects elder patients and it carries worse prognosis, as compared to GBM *IDH* mutant.[1]

Regardless of *IDH* mutational status, the current standard of treatment of GBM includes surgery, followed by chemotherapy with temozolomide and radiotherapy.[2] However, despite treatment, the most GBM patients undergo recurrence and die within 12–24 months, while only about 10% of them survive ≥5 years.[2,3]

A subset of GBMs have a silenced O-6-methylguanine-DNA methyltransferase (*MGMT*) gene, due to promoter methylation.[4] Since *MGMT* encodes for an enzyme which repairs DNA

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Address for correspondence: Prof. Valeria Barresi, Department of Diagnostics and Public Health, Polyclinic G.B. Rossi, P.le L.A. Scuro, 1, 37134 Verona, Italy.
E-mail: valeria.barresi@univr.it

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damage from alkylating agents, its expression is correlated to resistance to alkylating drugs, such as temozolomide.[4] On the other hand, *MGMT* promoter methylation is associated with higher sensitivity to temozolomide.[4] Thus, *MGMT* promoter methylation status is commonly assessed to predict responsiveness to temozolomide in GBM patients.

At present, no indication exists for the retesting (after initial tumor MGMT testing) of recurrent tumors after treatment. Indeed, contrasting results were reported on *MGMT* promoter methylation changing in GBM recurrence after chemoradiotherapy, with variable rates of change and conversion from methylated to unmethylated status and vice versa.[5-12]

The reasons for MGMT methylation change have been hypothesized to be the result of clonal selection during therapy, technical problems, and inadequacy of sampling, or tumor heterogeneity.

MGMT intratumor heterogeneity has been evaluated in several studies.[13-17] However, the main drawback of these studies was that the tissue was not preselected before the methylation analysis, and thus did not exclude areas of necrosis or inflammation.

In this study, we evaluated whether the change in *MGMT* promoter methylation during recurrence in GBM was due to initial tumor intratumor heterogeneity. We took samples from different concentric areas, including the periphery and central aspect of the tumor in 24 formalin-fixed and paraffin-embedded GBMs, and 11-paired posttreatment recurrences. Tumor samples used for methylation analysis were preselected for adequate cellularity and without evidence of necrosis or inflammation.

Materials and Methods

All procedures were performed in compliance with ethical standards and with Helsinki declaration principles. Patient's consent was obtained before the beginning of the study. Ethical issues were discussed with local ethics committee and it was decided that no formal approval was necessary. The study included 24 patients with surgically resected GBM (13 females and 11 male patients; age range: 42–73 years; mean age at diagnosis: 60.5 years). In all cases, the gross total resection was achieved. In the operation room, each tumor was subdivided into two different portions: peripheral (tumor portion adjacent to normal brain) and central, which were placed in different jars and fixed in formalin for 24 h at room temperature. Then, all samples were paraffin embedded and submitted for histological examination with hematoxylin and eosin (H and E) stain and immunohistochemical procedures.

Immunohistochemistry was performed using an automated immunostainer (Dako Autostainer Link 48 Instruments; Glostrup, Denmark) and the following antibodies against Olig-2 (clone 211F1.1, Cell Marque, Rocklin, CA, USA; 1:100), Glial Fibrillary Acidic Protein (clone 6F2; Dako, Glostrup, Denmark; 1:500), ATRX (Polyclonal; Life Science Sigma, St Louis, MO, USA; 1:750), IDH1 R132H (clone H09, Dianova, Gmbh, Germany; 1:200), p53 (clone DO-7, Glostrup, Denmark; 1:100), and Ki-67 (clone MIB-1, Glostrup, Denmark; 1:100).

In cases with negative IDH1 R132H stain, the mutational status of *IDH1*/2 genes was further evaluated by DNA sequencing. *IDH1*, *IDH2* genes were amplified by polymerase chain reaction (PCR) and both strands were sequenced using the ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). PCR conditions for *IDH1* and *IDH2* were the following: (1) initial denaturation step at 95°C for 5 min, (2) 40 cycles at 95°C/30 s, 58°C/30 s, and 72°C/30 s, and (3) a final step at 72°C/5 min. We used the following primers: *IDH1*-F CCATCACTGCAGTTGTAGGTT; *IDH1*-R GCAAAATCACATTATTGCCAAC; *IDH2*-F TGCAGTGGGACCACTATTATC; *IDH2*-R GTGCCCAGGTCAGTGGAT.

Thus, GBMs were subdivided into *IDH* mutant or *IDH* wild type. After surgery, all patients were submitted to concurrent radiotherapy and chemotherapy with temozolomide, according to the Stupp protocol.[2]

Follow-up data, including overall survival (OS) and recurrence-free survival, were available for all cases. Nineteen GBMs recurred during the follow-up time. In 11 cases, recurrent tumors were surgically resected. Surgical specimens were again split into two parts, peripheral and central, which were submitted to the same procedures described above.

O-6-methylguanine-DNA methyltransferase promoter methylation analysis

In each tumor specimen (peripheral or central), the areas with the highest number of tumor cells, and minimal amount of necrosis and inflammation were identified in a control H and E slide and marked by a pathologist. Those areas were manually dissected under microscopic guidance from the corresponding 20 μm section using a sterile blade and collected in a microtube. Finally, all samples (central and peripheral of each case) had at least 100 neoplastic cells, and a proportion between neoplastic cells and nonneoplastic contaminants >80%.

MGMT promoter methylation status was assessed by methylation-specific PCR (MS-PCR) using AlphaReal MGMT kit, which combines DNA extraction, DNA bisulfonation, and real-time PCR, according to the manufacturer instructions (Alphagenic Biotech Srl; Trieste, Italy).

Statistical analyses

The Fisher's exact test was used to analyze the statistical correlations between the intratumor heterogeneity or changing of MGMT promoter methylation status and the gender of the patients or *IDH* mutational status of the tumor.

The Mantel–Cox log-rank test was applied to assess the strength of association between OS and each of the parameters (age and gender of the patient, *MGMT* methylation status, *IDH*

mutational status, and intratumor heterogeneity) as a single variable FS.

Statistical analysis was performed using MedCalc 12.1.4.0 statistical software (MedCalc Software, Mariakerke, Belgium). $P < 0.05$ was considered statistically significant.

Results

Clinicopathological variables of GBMs in our cohort are shown in Table 1. Following immunohistochemistry and molecular analyses, GBMs were subdivided into 19 *IDH* wild-type (9 female and 10 male patients; age range: 50–73 years; mean age: 58.8 years) and 5 *IDH* mutant (4 female and 1 male patients; age range: 42–51 years; mean age: 46.4 years). All *IDH* mutant GBMs had R132H mutation; among them, two GBMs had originated from the progression of diffuse astrocytoma (DA), grade II. Thus, *MGMT* promoter methylation analysis was carried out in the preceding DA as well.

MGMT promoter methylation status was homogenous throughout the tumor (i.e., peripheral and central part of the tumor had the same methylation status) in 19 (80%) GBMs and heterogeneous (i. e., peripheral and central part of the tumor had different methylation status) in 5 (20%) [Figure 1].

Among homogeneous GBMs, 12 (63%) were unmethylated and 7 (37%) were methylated [Figure 2]. All 12 unmethylated GBMs were *IDH* wild-type. Among 7 methylated tumors, 4 were *IDH* wild-type and 3 *IDH* mutant.

In all five of the GBMs with heterogeneous *MGMT* promoter methylation status, the central part of the tumor was methylated and the peripheral part was unmethylated [Figure 3]. Two of five cases were *IDH* mutant GBMs, which had originated from the progression of DAs with an unmethylated *MGMT* promoter [Figure 3]. Three of the five cases were *IDH* wild-type.

Follow-up time ranged between 5 and 48 months. During this time, 18 cases recurred and 11 were submitted for surgical excision of the recurrent tumor. Time to progression ranged between 1 and 34 months. When we analyzed *MGMT* promoter methylation status in the recurrences, 8 (73%) cases were concordant with the primary tumor, while 3 (27%) were discordant [Figure 4]. In all three discordant cases, the primary tumor had heterogeneous *MGMT* promoter methylation status (peripheral part unmethylated and central part methylated) [Figure 5]. In one case, the initial tumor was an *IDH* mutant GBM, and its paired recurrent tumor was MGMT unmethylated [Figure 4], whereas, the other two cases were *IDH* wild-type GBMs, with recurrent MGMT methylated tumors [Figure 4].

Table 1: Clinicopathological characteristics and O-6-methylguanine-DNA methyltransferase promoter methylation status in 24 glioblastomas and in 11 paired recurrences

Case	Gender	Age	Site	MGMT promoter methylation	Recurrence	MGMT promoter methylation recurrence	IDH 1/2	DFS (months)	Status	FU (months)
1	Female	47	Temporal	UM/M*	Not		Mut	21	Alive	21
2	Female	63	Temporal	UM	Yes	UM	Wt	13	DOD	20
3	Female	73	Fronto-parietal right	M	Yes		Wt	3	Alive	22
4	Male	62	Frontal right	UM	Yes	UM	Wt	5	DOD	23
5	Female	70	Frontal left	UM	Yes	UM	Wt	14	Alive	36
6	Male	70	Occipital left	UM	Yes	UM	Wt	1	DOD	12
7	Male	61	Temporal left	UM	Yes		Wt	5	DOD	5
8	Male	64	Parieto-temporal left	UM	Yes		Wt	14	DOD	21
9	Female	69	Parietal left	UM/M*	Yes		Wt	6	DOD	6
10	Female	60	Frontal	M	Yes		Wt	12	DOD	22
11	Male	43	Temporal right	M	Not		Mut	20	Alive	20
12	Male	55	Rolandic right	M	Not		Wt	22	Alive	22
13	Male	66	Frontal	UM	Not		Wt	23	Alive	23
14	Female	54	Temporo-parietal right	M	Yes	M	Wt	27	DOD	27
15	Female	58	Frontal	M	Not		Mut	36	Alive	36
16	Female	42	Fronto-insular	M	Not		Mut	18	Alive	18
17	Male	54	Temporo-parietal right	UM	Yes	UM	Wt	14	DOD	26
18	Female	55	Fronto-temporal left	UM/M*	Yes	UM	Mut	34	Alive	48
19	Female	50	Temporal right	UM	Yes	UM	Wt	23	DOD	27
20	Female	57	Temporal left	UM/M*	Yes	M	Wt	23	Alive	31
21	Male	70	Frontal	UM	Yes		Wt	8	DOD	14
22	Female	60	Temporal left	UM/M*	Yes	M	Wt	16	DOD	25
23	Male	63	Fronto-parietal right	UM	Yes		Wt		DOD	14
24	Male	60	Frontal left	UM	Yes	UM	Wt	10	DOD	16

*The central part of the tumor was methylated and the peripheral one was unmethylated. DFS: Disease-free survival, FU: Follow-up, M: Methylated, UM: Unmethylated, Wt: Wild-type, Mut: Mutant, DOD: Died of disease, MGMT: O-6-methylguanine-DNA methyltransferase, IDH: Isocitrate dehydrogenase

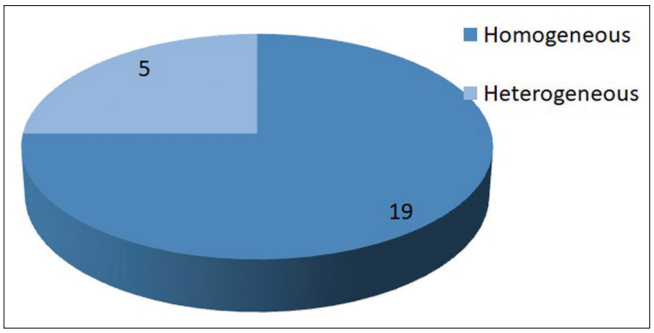

Figure 1: O-6-methylguanine-DNA methyltransferase promoter methylation status in 24 surgically resected GBMs

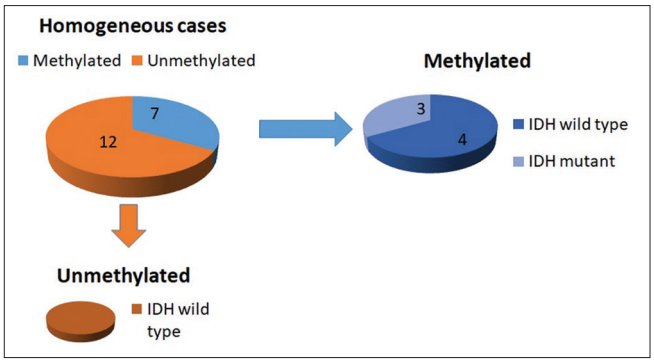

Figure 2: Glioblastomas with homogeneous O-6-methylguanine-DNA methyltransferase promoter methylation status

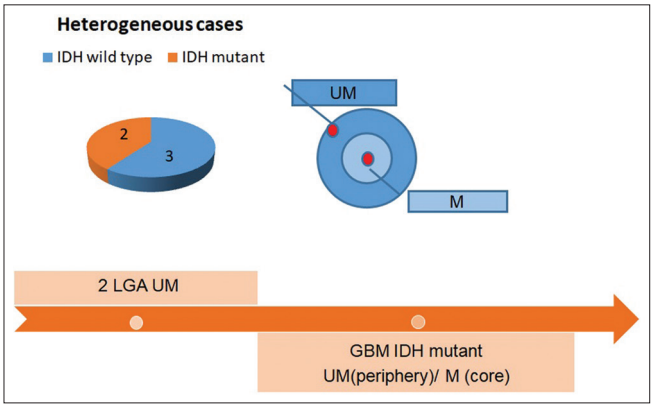

Figure 3: Glioblastomas with heterogeneous O-6-methylguanine-DNA methyltransferase promoter methylation status

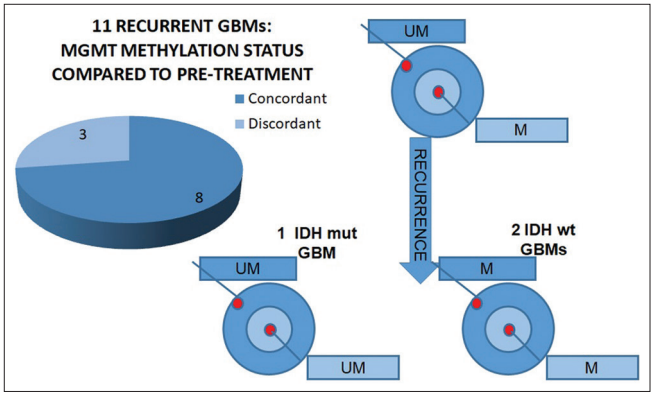

Figure 4: O-6-methylguanine-DNA methyltransferase promoter methylation status in 11 surgically resected glioblastoma recurrences

Intratumor heterogeneity was significantly more frequent in GBMs taken from female patients ($P = 0.0411$). The switching of *MGMT* promoter methylation status was not associated with any clinicopathological variables.

In univariate analysis, *IDH* mutation ($P = 0.0298$), *MGMT* promoter methylation ($P = 0.0115$), and female gender ($P = 0.0154$) were significantly associated with longer OS. Due to the small number of cases, we could not perform multivariate analyses. Among recurring cases, surgical resection of recurrent tumor was significantly associated with longer OS ($P = 0.0017$).

Discussion

Dynamic methylation changes of the *MGMT* promoter has been documented in several recurrent GBM studies but with different prevalences.[5-12] In this study, 27% of recurrent GBMs had different *MGMT* promoter methylation status compared to the corresponding primary tumor. Interestingly, discordance was observed only in cases having a primary GBM with heterogeneous *MGMT* methylation status. Therefore, it is tempting to speculate that the changes in *MGMT* promoter methylation status in recurrent GBMs may descend from heterogeneity of tumor cells in the initial tumor, with subsequent subclonal expansion in the recurrence.

Several studies previously analyzed intratumor heterogeneity of *MGMT* promoter methylation status in GBM and with conflicting results.[13-17] Parkinson *et al*.[13] reported that *MGMT* promoter methylation status is homogeneous across different samples of GBM. However, all samples they analyzed had been taken from the peripheral part of the tumors, and none from their central part.[13] In two other studies, *MGMT* promoter methylation heterogeneity was found in a small proportion of GBMs.[14,16] However, the authors claimed that heterogeneity could depend on the presence of high number of nontumor contaminants in the unmethylated parts of the tumors.[14,16]

Interestingly, Della Puppa *et al*.[17] found intratumor heterogeneity of *MGMT* promoter methylation in 33.3% of GBMs. In their study, *MGMT* methylation analysis was carried out in different concentric samples of GBMs.[17] Indeed, they hypothesized that tumor stem cells, which are the most resistant to temozolomide, mainly reside in the central part of the tumor.[18] In their heterogeneous samples, *MGMT* promoter methylation status was different in the intermediate part of the tumor compared to the peripheral and inner parts.[18] However, methylation analysis had been carried out on frozen samples, with no histological verification, and eventual exclusion of nonneoplastic contaminants.[18]

In this study, we found *MGMT* promoter methylation at the center, but not at the periphery of the tumor, in 20% of GBMs. Heterogeneity could not be related to sample inadequacy. Indeed, methylation analysis was performed on

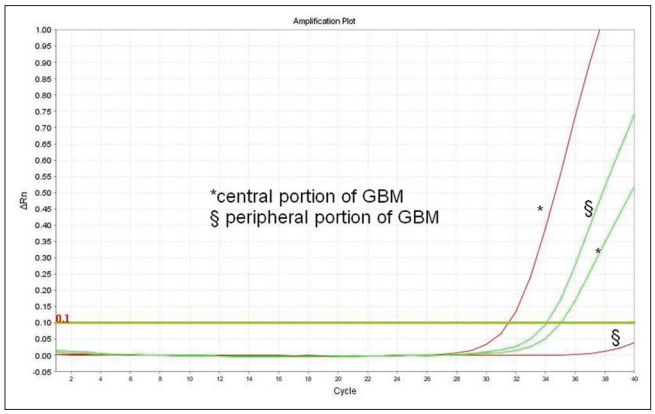

Figure 5: Polymerase chain reaction amplification plots relative to a heterogeneous glioblastoma, with methylation changes occurring at the recurrence. The red curves refer to amplification of promoter methylated O-6-methylguanine-DNA methyltransferase, while the green ones refer to the amplification of promoter unmethylated O-6-methylguanine-DNA methyltransferase. In the primary tumor, the central part was methylated and peripheral one was unmethylated

histologically verified neoplastic samples, with selected areas having adequate number of neoplastic cells and low amount of nontumor contaminants. Interestingly, two of the heterogeneous cases were *IDH* mutant GBMs, which had originated from the progression of LGAs. Since the corresponding LGAs were unmethylated, we can suppose that *MGMT* methylation was acquired in a subclone of tumor cells during progression, thus leading to heterogeneous methylation status in the secondary GBM. This is intriguing given that *IDH* mutations have been shown to induce extensive DNA methylation in gliomas.[19] Accordingly, all the homogeneously unmethylated GBMs in our cohort were *IDH* wild type tumors.

To the best of our knowledge, only one study in LGAs investigated *MGMT* methylation status in relation to IDH mutational status.[20] In that study, the majority of *IDH* mutant LGAs had *MGMT* promoter methylation,[20] in accordance with the hypermethylated status of *IDH* mutant tumors.[19] However, a small proportion of *IDH* mutant astrocytomas had unmethylated *MGMT* and this condition was associated with worse prognosis,[20] similarly to that observed in our cases, which progressed to GBM.

In our cohort of patients, *MGMT* methylation and *IDH* mutation were significantly associated with longer OS, which confirm their relevant prognostic value in GBM patients.[1,21] In addition, female gender was a favorable prognostic factor in our cohort, similarly to that reported in the study by Franceschi *et al*.[22] Interestingly, among recurring tumors, surgery of recurrences increased the OS, which is in line with the findings in other studies.[23,24]

Conclusion

This study confirms that *MGMT* methylation status can vary in recurrent GBM, and that it can change from methylated to unmethylated and vice versa in comparison to the original tumor. The fact that MGMT promoter methylation can change from initial tumor to recurrent tumor, raises the question of whether retesting of recurrent tumors is needed for therapeutic decisions. For instance, if the initial tumor is unmethylated but at recurrence becomes methylated, would a rechallenge with temozolomide be indicated? According to our results, *MGMT* variation may depend on intratumor heterogeneity in the primary GBM, which can be observed in both *IDH* mutant and *IDH* wild type tumors. Since *MGMT* promoter methylation status seems to vary according to a spatial criterion, further studies on larger cohorts are needed to clarify the site from which, the optimal tumor specimen for methylation analysis should be taken.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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