

## Variation of O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT) promoter methylation in serial samples in glioblastoma

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**Abstract** Methylation of the promoter region of the *O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT)* gene is known to be predictive of response to temozolomide treatment in patients with glioblastoma. Contrastingly, little is known about variation in the methylation status of the *MGMT* promoter after treatment or across different regions of the same tumor. About 22 samples from 10 patients who had undergone multiple resections of a glioblastoma were examined with promoter sequencing. Of these, 20 were also analyzed using Methylation Specific PCR (MSP). The methylation status of the *MGMT* promoter was altered in the specimens obtained pre and post treatment in 2 of 9 samples as assessed by MSP and 7 out of 10 patients as assessed by promoter sequencing. In four patients, the *MGMT* promoter was unmethylated at primary surgery, but displayed some methylation (32, 44, 12, and 4%) on post-treatment sampling. Alteration in MSP status from unmethylated to methylated was also observed in 2 of these 4 patients. In another patient, methylation increased from

40% on initial sampling to 68% on the second sample. The remaining two patients initially demonstrated some degree of methylation (72% and 12%); subsequent sampling showed no methylation of the *MGMT* promoter. To ensure variable methylation status was not due to intra-tumoral variability, three to four specimens were sampled from different regions of large glioblastomas ( $n = 7$ ). Promoter sequencing revealed minimal variation in methylation in all but two sites examined. Immunohistochemistry also demonstrated minimal change in *MGMT* expression across the tumors. This suggests that variation in *MGMT* promoter methylation can occur within the same tumor after treatment, necessitating caution in clinical decision-making based on this analysis.

**Keywords** Glioma · MGMT · Temozolomide · Methylation · DNA repair

### Introduction

Glioblastoma, the most common primary brain tumor in adults, is a rapidly progressive and universally fatal disease [1]. Standard therapy for these tumors has been surgery followed by radiotherapy, based predominantly on the findings of a randomised controlled trial conducted in the late 1970s which demonstrated improved survival with radiotherapy [2]. The role of chemotherapy has been controversial with no single agent or regime gaining acceptance despite a recently published metaanalysis of 12 studies demonstrating some benefit [3]. However a recent study demonstrated an improvement in median survival from 12.1 months to 14.6 months with the addition of temozolomide to the previous standard of surgery and radiotherapy. Temozolomide, an alkylating agent, causes

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abnormal DNA repair by causing methylation of the O<sup>6</sup> position of guanine residues [4, 5].

The DNA repair enzyme O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT) is responsible for removal of an alkyl group from the O<sup>6</sup> position of guanine, and plays an instrumental role in DNA repair [6]. MGMT protein expression occurs in normal tissues and many cancers, however the level of expression varies widely [7–10]. The *MGMT* gene has been well characterized, and is located on chromosome 10q26. Epigenetic silencing of *MGMT* expression by promoter methylation has been demonstrated to be a common event in human neoplasia including gliomas [7, 10]. Methylation of the *MGMT* promoter region can also result in increased formation of mutations in cancer [11]. When MGMT expression is silenced, O<sup>6</sup>-methylguanine is able to pair with thymine during DNA replication, resulting in conversion of guanine–cytosine to adenine–thymine pairs in DNA. Crosslinking may also occur between the O<sup>6</sup>-methylguanine-DNA adduct and opposite cytosine residues, thereby blocking DNA replication [10].

In the case of glioblastoma, hypermethylation of the *MGMT* promoter is of interest for the role it plays in effectiveness of alkylating agent chemotherapy as much as for its role in tumorigenesis. As the major mechanism of action of MGMT is in direct opposition to that of temozolomide, the expression of MGMT may be an important factor when considering sensitivity of patients with glioblastoma to temozolomide therapy. A number of studies have demonstrated the value of assessment of the methylation status of the *MGMT* gene in predicting the response of patients to therapy with alkylating agents [12–16]. More recently Hegi and colleagues demonstrated that methylation of the *MGMT* gene in patients with glioblastoma resulted in improved survival after temozolomide treatment when compared with those patients in which the *MGMT* promoter was unmethylated (21.7 vs. 12.7 months) [5]. This study demonstrated the potential importance of *MGMT* promoter methylation when considering which patients with glioblastoma should be treated with temozolomide, that is can we consider *MGMT* promoter methylation as a potential predictive marker for treatment benefit, thus potentially allowing to select patients for specific treatments.

A number of methods have been described to assess the methylation status of the promoter of the *MGMT* gene. The most commonly used method is Methylation Specific PCR (MSP). This involves the use of two sets of primers, one specific for an unmethylated promoter and one specific for a methylated promoter. The presence of a PCR product with either set of primers indicates a positive result. The number of CpG dinucleotides assessed by either set of primers is typically small, usually around 5 in

number. An alternative method of assessment of the methylation status of the *MGMT* promoter is by promoter sequencing. This involves amplification of a region of the *MGMT* promoter rich in CpG dinucleotides to determine the methylation status of the promoter. Due to the use of primers that frame a larger region of the promoter, this technique allows the interrogation of a larger number of CpG dinucleotides, typically around 25. To date only one group has applied the latter technique to glioma—in oligodendroglioma [17] and more recently in high grade astrocytoma [18].

Furthermore, there are no studies examining potential alterations in the methylation status of the *MGMT* promoter in recurrent GBM tumor(s) when compared directly to a matched primary GBM tumor (from the same patient) to ascertain the effect of exposure to radiotherapy and chemotherapy agents on gene expression. In this study we examined *MGMT* promoter methylation and protein expression in 10 patients where matched primary and recurrent frozen glioblastoma tissue were available. We also measured the level of *MGMT* promoter methylation and protein expression in multiple sampling sites taken from large glioblastoma tumors ( $n = 7$ ). Our study demonstrates that *MGMT* promoter methylation varies in tumor samples from the same patient before and after treatment taken at a mean interval of 207.38 days (range 19–367), and that this is unlikely to be the result of tumor heterogeneity.

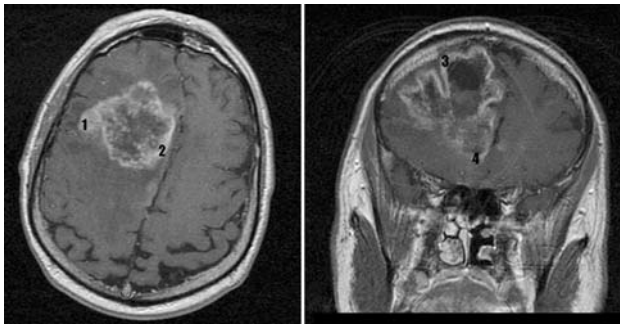
## Materials and methods

### Tumor collection

Tumor samples were collected from a total of 16 patients undergoing surgery at the Royal North Shore and North Shore Private Hospitals. In 10 patients, tumors were collected from an initial resection and a later resection. In 2 of these 10 patients, tumors were collected on a total of 3 occasions. Additionally, in 7 patients, 3 or 4 samples were taken from the same large glioblastoma (Fig. 1). The clinical information relating to these patients is summarized in Table 1. Approval for this study was obtained from the Northern Human Research Ethic Committee (NHREC). Tumors were collected at the time of resection, snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

### DNA extraction and bisulfite modification

DNA was extracted from homogenized tumor specimens using standard phenol-chloroform extraction methods.



**Fig. 1** T1 weighted Gadolinium enhanced MRI image of a glioblastoma from which four samples were taken for analysis. Numbers represent the sites from which tumour was taken. This was histologically confirmed to be glioblastoma without significant areas of necrosis

**Table 1** Patient clinical information

| Patient | Age | Sex | Tumor location | Interval between surgeries (days) | Interval treatment          |
|---------|-----|-----|----------------|-----------------------------------|-----------------------------|
| A       | 62  | M   | Right Temporal | 237                               | R, T                        |
| B       | 58  | M   | Right Temporal | 19                                | Nil                         |
| C       | 47  | M   | Left Frontal   | 113                               | R, T                        |
| D       | 51  | M   | Left Parietal  | 296 <sup>a</sup>                  | R, T, C                     |
| E       | 46  | M   | Right Temporal | 175                               | R, T                        |
| F       | 52  | M   | Right Parietal | 329                               | R, T, P                     |
| G       | 35  | F   | Left Parietal  | 367                               | T                           |
| H       | 68  | F   | Left Temporal  | 206                               | R, T                        |
| I       | 59  | M   | Left Parietal  | 182 <sup>a</sup>                  | R, T, P                     |
| J       | 60  | M   | Left Temporal  | 213                               | R, T, C, Th, E, Iso, Iri, B |

Treatment Abbreviations: R, Radiotherapy; T, Temozolomide; C, Celecoxib; P, Procarbazine; Th, Thalidomide; E, Erlotinib; Is, Isotretinoin; Ir, Irinotecan; B, Bevacizumab

<sup>a</sup> Patients D and I had three surgical resections. The interval is the mean interval between each of the procedures

Commercial methylated and unmethylated DNA (CpGenome™, Chemicon International, Temecula, California, USA) was obtained for use as internal controls.

**Table 2** PCR primers and product sizes

| Purpose             | Primer                                | Product (bp) |
|---------------------|---------------------------------------|--------------|
| MSP                 |                                       |              |
| Methylated          | Fw 5'-TTTCGACGTTCCGTAGGTTTTTCGC-3'    | 81           |
|                     | Rv 5'-GCACTCTTCCGAAAACGAAACG-3'       |              |
| Unmethylated        | Fw 5'-TTGTGTTTTGATGTTGTAGGTTTTTGT-3'  | 93           |
|                     | Rv 5'-AACTCCACACTCTTCCAAAAACAAAACA-3' |              |
| Promoter sequencing |                                       |              |
| 1st PCR             | Fw 5'-ATGTTGGGATAGTTCGCGTTTTTAGA-3'   | 372          |
|                     | Rv 5'-ACCTAAAACCTAACACCTCCCCCT-3'     |              |
| 2nd PCR             | Fw 5'-ATGTTGGGATAGTTCGCGTTTTTAGA-3'   | 317          |
|                     | Rv 5'-CCAATCCACAATCACTACAAC-3'        |              |

bp, Base pairs; MSP, Methylation Specific PCR; Fw, Forward; Rv, Reverse

Extracted tumor or control DNA (1 µg) was denatured with sodium hydroxide (0.3 M). Bisulfite modification was performed using the MethylEasy kit (Human Genetic Signatures Pty Ltd, Macquarie Park, NSW, Australia), according to the manufacturer's instructions. Bisulfite treatment converts unmethylated cytosine residues to uracil, while those cytosine residues that are methylated are protected.

Methylation Specific PCR (MSP)

Methylation Specific PCR of the *MGMT* promoter was performed using bisulfite treated DNA (2 µl). The PCR included Enhancer (10%) (Invitrogen Corporation, Carlsbad, California, USA). Forward and reverse primers (0.5 µM) were adapted from Mollemann et al [17] and are listed in Table 2. The annealing temperature for both methylated and unmethylated MSP reactions was 57°C, and the reaction was carried out for 30 cycles.

PCR amplicons were separated on non-denaturing 4% polyacrylamide gels, stained with Ethidium Bromide and visualized using a Fujifilm FLA 3000 Fluorescent Image Analyzer (Fuji Photo Film Co., Ltd, Tokyo, Japan). Analysis of the MSP was performed blinded to all other information including clinical data and the results of promoter sequencing (see below).

Promoter sequencing

Sequencing of 25 CpG dinucleotides in the *MGMT* promoter was performed using a semi-nested approach, whereby the forward primer was used for both rounds of the PCR. Bisulfite treated genomic DNA (2 µl) was added to the Enhancer (Invitrogen, California, USA) boosted PCR mix together with primers listed in Table 2 with an annealing temperature of 42°C and with the reaction being carried out for 30 cycles. The resultant first round PCR

product (1  $\mu$ l) was then used in the second round PCR using primers as listed in Table 2 for 30 cycles with an annealing temperature of 59°C.

PCR amplicons were purified using the Montage PCR Device system (Millipore Corporation, Bedford, Massachusetts, USA) according to the manufacturer's instructions. Sequencing was performed in forward and reverse directions at Sydney University Prince Alfred Macromolecular Analysis Centre (SUPAMAC) using the same primers as those used for the second round PCR (see Table 2).

A total of 25 CpG dinucleotides in the *MGMT* promoter were examined, and compared to control samples. The extent of methylation was measured as a percentage of total methylation. Sequencing of all samples was repeated at least once. Samples from each patient were repeated in the same assay.

#### Multiple tumor site samples

To examine the possibility of variation within the same tumor, multiple specimens were taken from the tumor during a single surgery in seven patients. In all but one patient (patient I) the surgery was the first resection. The specimens were taken from tumors of a minimum of 6 cm diameter, and from four different peripheral macroscopically abnormal regions, as determined by a neurosurgeon (JFP). An example is shown in Fig. 2. These sites were subsequently confirmed to be glioblastoma on histological examination. DNA from all sites was extracted as described above. The DNA was subsequently bisulfite treated and the *MGMT* promoter analyzed as described above. A section of each site within the tumor was also taken, fixed with formalin and paraffin embedded for histological examination and immunohistochemistry.

#### MGMT immunohistochemistry

Immunohistochemistry for MGMT was performed on formalin fixed paraffin embedded tissue using a mouse monoclonal antibody (Clone MT23.2, Affinity Bioreagents CO USA). A single representative block from each tumor was sectioned at 4  $\mu$ m onto positively charged slides (Ultrafrost plus, Menzel-Glaser, Germany). Tissue was available for immunohistochemistry in 18 of the 22 samples. Slides were then stained using the Vision Biosystems bondMax autostainer (Vision Biosystems, Mount Waverley, Victoria, Australia) according to the manufacturer's protocol. Briefly this involved slides being dewaxed in Bond Dewax solution (Vision Biosystems, Mount Waverley, Victoria, Australia) and hydrated in Bond Wash solution (Vision Biosystems, Mount Waverley, Victoria, Australia). Antigen retrieval was performed at acidic pH using Epitope Retrieval 1 solution (Vision Biosystems, Mount Waverley, Victoria, Australia) for 30 min at 100°C. Slides were then incubated with the primary antibody at a concentration of 1:8,000 for 30 min at room temperature. Antibody detection was performed using the biotin free Bond Polymer Defined Detection System (Vision Biosystems, Mount Waverley, Victoria, Australia) according to the manufacturer's protocol. Slides were then counterstained with haematoxylin. External positive and negative controls (tonsillar tissue with known areas of positive and negative staining) were examined with each batch of slides. Additionally, endothelial cells and lymphocytes served as internal positive controls. An independent pathologist who was blinded to molecular and clinical data examined slides. Slides were scored semiquantitatively based on positive nuclear staining—0 (negative), 1 (approximately <10% staining), 2 (10–25% staining) to 3 (>25% staining).

**Fig. 2** Representation of the methylation pattern of tumors taken pre and post treatment. Columns represent CpG dinucleotides. A shaded square represents methylation at that site

| Patient | Resection       | CpG Dinucleotide |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  |
|---------|-----------------|------------------|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|--|
|         |                 | 1                | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 |  |
| A       | 1 <sup>st</sup> |                  |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  |
|         | 2 <sup>nd</sup> |                  |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  |
| B       | 1 <sup>st</sup> |                  |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  |
|         | 2 <sup>nd</sup> |                  |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  |
| C       | 1 <sup>st</sup> |                  |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  |
|         | 2 <sup>nd</sup> |                  |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  |
| D       | 1 <sup>st</sup> |                  |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  |
|         | 2 <sup>nd</sup> |                  |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  |
|         | 3 <sup>rd</sup> |                  |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  |
| E       | 1 <sup>st</sup> |                  |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  |
|         | 2 <sup>nd</sup> |                  |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  |
| F       | 1 <sup>st</sup> |                  |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  |
|         | 2 <sup>nd</sup> |                  |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  |
| G       | 1 <sup>st</sup> |                  |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  |
|         | 2 <sup>nd</sup> |                  |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  |
| H       | 1 <sup>st</sup> |                  |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  |
|         | 2 <sup>nd</sup> |                  |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  |
| I       | 1 <sup>st</sup> |                  |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  |
|         | 2 <sup>nd</sup> |                  |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  |
|         | 3 <sup>rd</sup> |                  |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  |
| J       | 1 <sup>st</sup> |                  |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  |
|         | 2 <sup>nd</sup> |                  |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |  |

**Results**

Analysis of sequential samples

DNA was successfully extracted from tumor samples obtained from ten patients with matched primary and recurrent samples to evaluate the effect of treatment on MGMT status. For eight patients, a matched primary and one recurrent tumor was available for analysis, whilst for two patients, a matched primary and two recurrent tumors were available for MGMT promoter methylation analysis. MGMT promoter sequencing was successfully performed in all 22 samples and 20 samples were also subjected to MSP analysis (2 samples did not amplify after 3 attempts). The results are summarized in Table 3. Sequencing analysis results are demonstrated in Fig. 2. Both MSP and promoter sequencing confirmed an unmethylated MGMT promoter status that was unchanged in the primary and recurrent tumors from three patients (Patients F, H and J—Table 3). In four patients (patients A, B, C and I) the MGMT promoter was initially unmethylated in the primary tumors by promoter sequencing, but interestingly displayed a degree of methylation in the recurrent tumors (32, 44, 12 and 4% respectively). This was also accompanied by a change in MSP from unmethylated to methylated in 2 of the 4 patients. In another patient (Patient G) the percentage of methylation increased from 40% methylation on the initial primary sample to 68% in the recurrent tumor. By contrast, the remaining two patients (patients D and E) initially demonstrated some degree of methylation (72% and 12% respectively), however their recurrent tumors showed no methylation.

The results of immunohistochemistry are included in Table 3. Immunohistochemistry was successful in all samples where tissue was available. There was a good correlation between the increasing MGMT promoter methylation and decreased MGMT expression. In most patients who had been treated with temozolomide, there

was decreased MGMT expression in the post treatment sample when compared to the primary sample.

Analysis of intra-tumoral specimens

The results of MGMT promoter methylation analysis of multiple sites within the same tumor are summarized in Table 4. In general, the degree of methylation was homogenous across the tumors. There were however some exceptions—one tumor (tumor 1) displayed 16% methylation in one site with 0% in the other 3; another (tumor 4) displayed 24, 4 and 0% across the three sites successfully assessed. Similarly at the protein level as assessed by immunohistochemistry, MGMT expression was fairly constant across the tumours. There was one notable exception to this—one site in tumor 4 displayed low protein expression corresponding to promoter methylation as assessed by both sequencing and MSP (Fig. 3).

**Discussion**

DNA repair enzymes play a vital role not only in the pathogenesis of cancer, but also in the effectiveness of treatment. The methylation status of the MGMT promoter, and subsequent silencing of MGMT production and activity, may have an important role in the effectiveness of alkylating chemotherapeutic agents such as temozolomide [11]. It has been demonstrated by Hegi and colleagues that methylation of the MGMT promoter, as assessed by MSP, is predictive of patient response to temozolomide [5].

An alternative method of assessing the methylation status of the MGMT promoter is sequencing of bisulfite treated genomic DNA. Bisulfite treatment results in the conversion of unmethylated cytosine residues to uracil, while methylated cytosines are protected. By amplification with PCR and sequencing, it is possible to assess the

**Table 3** Analysis of sequential samples

| Patient | 1st sample |     |     | 2nd sample |     |     | 3rd sample |     |     |
|---------|------------|-----|-----|------------|-----|-----|------------|-----|-----|
|         | MSP        | SEQ | IHC | MSP        | SEQ | IHC | MSP        | SEQ | IHC |
| A       | U          | 0   | 3   | M          | 32  | 1   |            |     |     |
| B       | U          | 0   | NA  | M          | 44  | 0   |            |     |     |
| C       | U          | 0   | 1   | U          | 12  | NA  |            |     |     |
| D       | NA         | 72  | 2   | U          | 0   | 3   | U          | 0   | 3   |
| E       | U          | 12  | NA  | U          | 0   | NA  |            |     |     |
| F       | U          | 0   | 1   | U          | 0   | 2   |            |     |     |
| G       | NA         | 40  | 1   | M          | 68  | 0   |            |     |     |
| H       | U          | 0   | 1   | U          | 0   | 0   |            |     |     |
| I       | U          | 0   | 1   | U          | 4   | 2   | U          | 4   | 1   |
| J       | U          | 0   | 3   | U          | 0   | 3   |            |     |     |

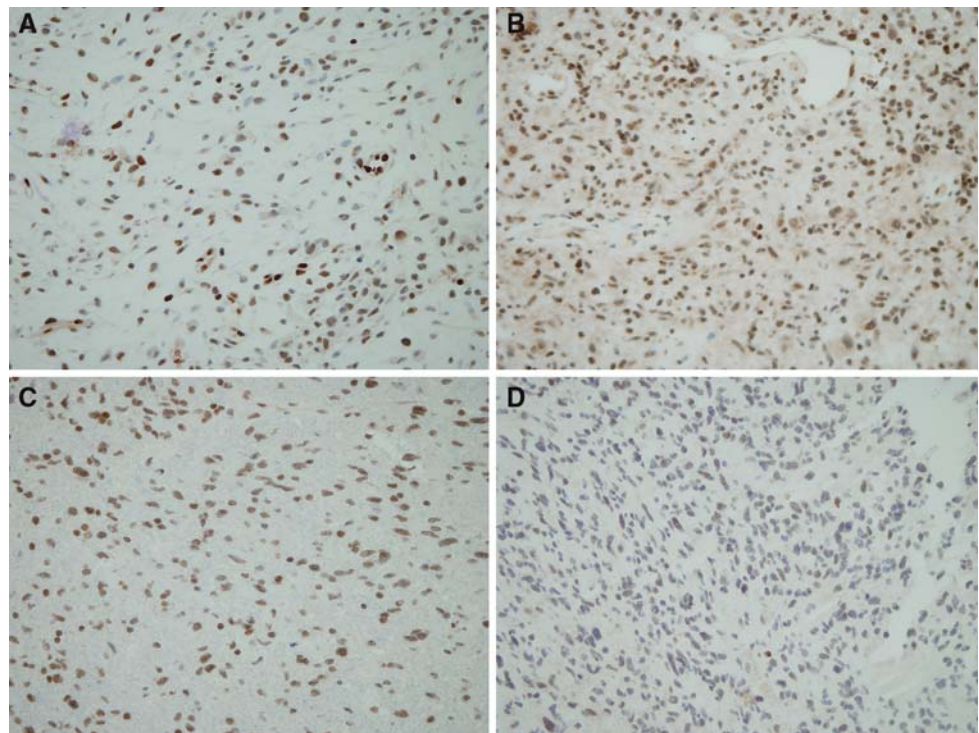
MSP, Methylation Specific PCR; U, Unmethylated; M, Methylated; NA, Not available; SEQ, Promoter Sequencing. Expressed as percentage of 25 CpG dinucleotides assessed; IHC, Immunohistochemistry. Four tiered scale from 0 (no nuclear staining) to 3 (diffuse strong positive staining)

**Table 4** Analysis of intra-tumoral sampling

| Tumor | 1st location |     |     | 2nd location |     |     | 3rd location |     |     | 4th location |     |     |
|-------|--------------|-----|-----|--------------|-----|-----|--------------|-----|-----|--------------|-----|-----|
|       | SEQ          | MSP | IHC | SEQ          | MSP | IHC | SEQ          | MSP | IHC | SEQ          | MSP | IHC |
| 1     | 0            | U   | 0   | 0            | U   | 1   | 0            | U   | 1   | 16           | U   | 0   |
| 2     | 0            | U   | 3   | 0            | U   | 2   | 12           | U   | 3   | 0            | U   | 3   |
| 3     | 0            | U   | 0   | 4            | NA  | 1   | 4            | U   | 0   | 4            | U   | 2   |
| 4     | 0            | U   | 3   | 24           | M   | 1   | NA           | U   | 3   | 4            | U   | 3   |
| 5     | 0            | U   | 3   | 8            | U   | 3   | 0            | U   | 3   | 0            | U   | 3   |
| 6     | 0            | U   | 3   | 4            | U   | 3   | 0            | U   | 3   | 0            | U   | 3   |
| 7     | 0            | U   | 3   | 0            | U   | 3   | 0            | U   | 3   |              |     |     |

MSP, Methylation Specific PCR; U, Unmethylated; M, Methylated; NA, Not available; SEQ, Promoter Sequencing. Expressed as percentage of 25 CpG dinucleotides assessed; IHC, Immunohistochemistry. Four tiered scale from 0 (no nuclear staining) to 3 (diffuse strong positive staining)

**Fig. 3** Example of MGMT immunohistochemistry performed on sections from four sites within the same tumor (tumor 4). Three of the four sites (A, B, C) showed strongly positive nuclear staining. Weaker staining was seen in the fourth site (D). This corresponded with the findings of promoter methylation analysis—sites A, B and C were unmethylated on both MSP and sequencing, whereas site D was methylated on MSP and 24% methylated on sequencing



methylation status of a far wider region of the promoter than MSP. In our case, we could assess 25 CpG dinucleotides whereas primers for MSP generally contain around 5 CpG dinucleotides. Promoter sequencing of the *MGMT* gene has previously been described in gliomas, but only by one group on two occasions [17, 18]. The downregulation of MGMT expression by promoter methylation appears to be gradual and related to the degree of methylation as assessed by promoter sequencing based on a recent in vitro investigation [19].

Our study has demonstrated that the extent of methylation of the *MGMT* promoter may either increase or decrease between different specimens taken from the same tumor before and after adjuvant treatment. There are a

number of potential explanations for these changes, including regional variation within the tumor, direct influence on methylation by treatment, selection of unmethylated cell populations by treatment and further dedifferentiation of tumor.

Glioblastomas are known to be heterogenous at not only the macroscopic and microscopic levels but also at the molecular level [1, 20, 21]. The most well recognized implication of this heterogeneity is sampling error that can occur at the time of histological diagnosis. Additionally, the application of immunohistochemical markers such as proliferative markers may be affected by heterogeneity as has been described [22]. More recently, heterogeneity of molecular markers (specifically loss of heterozygosity of

chromosomes 1p and 19q) has been demonstrated [23]. In melanoma, a similarly heterogenous tumour, variability in *MGMT* promoter methylation has been demonstrated [24].

By obtaining tissue from different regions of a number of large glioblastomas we were able to demonstrate that for the most part there was little heterogeneity in *MGMT* promoter status across each tumor. Similarly, there was little variability in *MGMT* protein expression across each tumor. This suggests that the variation seen between samples from consecutive surgeries is unlikely to represent variation within the one persistent or recurrent tumor. This confirms the findings of a recent study [18].

By contrast, Patient B in our series can be considered an example of regional variation within the same tumor. This patient had evidence of multifocal disease at presentation, and the largest region of tumor was debulked. When another region expanded rapidly less than 3 weeks later, a second operation was performed. This appeared to be rapid progression rather than tumor recurrence. While the *MGMT* promoter was completely unmethylated in the first tumor sample, the second demonstrated 44% methylation.

Pharmacological manipulation of methylation in cancer may have important implications for treatment. Indeed specific demethylating agents such as 5-aza-2'-deoxycytidine have been developed [25]. What is less clear is the impact of other treatments including radiotherapy. Widespread changes in DNA methylation have been demonstrated in some in vitro studies, however in one such study the *MGMT* promoter was not demonstrated to be affected by radiotherapy [26, 27]. On the other hand, microscopic changes after radiotherapy in glioblastoma have been well recognized. So while there is as yet no evidence of alteration of *MGMT* promoter methylation after radiotherapy, it is possible that some of the changes observed in our study are attributable to radiotherapy.

As demonstrated in Table 1, the majority of patients were treated with alkylating agent chemotherapy in the interval between surgeries. It would be expected that tumor cells within the greater tumor population with a methylated *MGMT* promoter, and thus silencing of *MGMT* expression, would be more sensitive to this form of treatment. These cells may be killed by the treatment, while those relatively resistant cells with an unmethylated *MGMT* promoter may survive. Analysis of the *MGMT* promoter in patient E where the tumor initially showed some methylation (12%) but on a later sample was entirely unmethylated may reflect this. Additionally, the *MGMT* protein acts in a suicide manner, that is it may only remove an alkyl group from the O<sup>6</sup> position of guanine on one occasion prior to degradation [28, 29]. *MGMT* protein may be depleted by alkylating agent treatment, and as reflected by immunohistochemistry, without alteration in methylation status. Indeed this property has recently been exploited in clinical

trials utilising metronomic temozolomide dosing regimes [30].

As tumors develop, regions of both hypermethylation and hypomethylation are known to develop [31, 32]. As a tumor progresses, irrespective of treatment, the inevitable changes in methylation state that occur may have been reflected in the results demonstrated above. For example, the progression in patient A from glioblastoma to gliosarcoma may have been associated with the development of a generalised hypermethylated state, with methylation of tumor suppressor genes leading to increased tumor aggressiveness despite a potential increased sensitivity to temozolomide which methylation of the *MGMT* promoter could confer. This may explain why a tumor may become more methylated after radiotherapy and alkylating agent chemotherapy.

The presence of normal tissue in a tumor sample may have some impact on methylation assessment. As most normal astrocytes, microglia, lymphocytes and endothelial cells generally have an unmethylated *MGMT* promoter this may result in an underestimation of methylation in a tumor. This could manifest as an apparent change in methylation status if there were varying degrees of “normal tissue contamination” in two or more samples. The extent of inflammation varies widely between various glioblastomas and the presence of DNA from inflammatory cells is difficult to control for in methylation analysis.

This study confirms that *MGMT* promoter methylation is largely homogenous across glioblastomas. While the value of *MGMT* promoter methylation as a predictive marker has been demonstrated at initial biopsy, the findings of our study suggest that after treatment it may be necessary to repeat this analysis if further treatment decisions are to be based on methylation analysis.

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