

Somatic mutations in glioblastoma are associated with methylguanine-DNA methyltransferase methylation

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Abstract. The high level of methylguanine-DNA methyltransferase (MGMT) in glioblastoma is responsible for resistance to alkylating agents, such as temozolomide (TMZ). In glioblastomas with a methylated *MGMT* promoter, MGMT deficiency is presumed, resulting in an enhanced effect of TMZ. The aim of the present study was to investigate whether genomic alterations work synergistically with *MGMT* methylation status and contribute to the response to treatment and overall prognosis in glioblastoma. The current study included a cohort of 35 glioblastoma patients, with *MGMT* promoter methylation present in 48% of tumors. *MGMT* methylation was associated with significantly longer median survival (29.0 months) compared with patients without *MGMT* methylated tumors (12.0 months), as well as longer median time to progression following TMZ treatment (13.2 months, compared with 5.6 months for patients with an unmethylated *MGMT* status). In addition, somatic variants in hot spot exonic regions of 50 key cancer genes were examined in these glioblastomas. Non-synonymous mutations in methylated *MGMT* glioblastomas were four times higher compared with unmethylated *MGMT* glioblastomas. Furthermore, significantly increased frequencies of mutations in the *TP53*, *CDKN2A*, *PTEN* and *PIK3CA* genes were detected in *MGMT* methylated glioblastomas. The relative significance of these mutations, and their contribution to TMZ sensitivity, adjunct to *MGMT* methylation, require further investigation in a larger cohort.

Introduction

Glioblastoma (GBM) is the most common type of brain tumor, with high mortality rates. The aggressive biology of GBM and resistance to therapy are major clinical problems for patients diagnosed with the disease (1). At present, standard treatment includes maximal safe surgery, concomitant radiotherapy and temozolomide (TMZ) chemotherapy, where a median survival time of <15 months is observed in patients fit for this current first-line therapy (1). In terms of years of life lost, the population burden from GBM is the highest of all malignancies (2). GBM frequently affects young people (30-40 years of age), as well as the elderly (3). Physically or cognitively disabled patients require care from the early stages of the disease, which results in increased expenses. Despite advances in therapeutic strategies, no significant reductions in the age-standardised incidence and mortality rates of this disease have been observed within the last decade (3).

Patients with a good Eastern Cooperative Oncology Group performance status or Karnofsky score at the time of diagnosis undergo surgical resection (4,5). Complete removal of the tumor is challenging due to the infiltrative nature of GBM; however, 80-95% debulking is typically achieved unless the disease is in a neurologically eloquent location (6). The standard treatment, known as 'The Stupp Protocol', consists of concurrent temozolomide (TMZ) therapy and radiotherapy (60 Gy/30 fractions), followed by subsequent adjuvant TMZ therapy for 6-12 cycles (1). TMZ functions by alkylating the guanine residues in DNA, resulting in mispairing with thymine during DNA replication and cellular arrest in rapidly dividing cells (7,8).

Methylguanine-DNA methyltransferase (MGMT) removes mutagenic alkyl adducts and, thus, protects DNA from the damage induced by TMZ and other alkylating agents. Loss of MGMT expression commonly occurs in human neoplasia (9). The expression of MGMT is regulated by hypermethylation of the CpG islands within the promoter and enhancer regions of the gene (10-12). MGMT deficiency is presumed in GBMs with a methylated *MGMT* promoter, resulting in the enhanced cytotoxicity of TMZ (13). Studies have demonstrated that presence of *MGMT* promoter methylation is correlated with the response of patients to treatment with TMZ; thus, it is considered to be a predictive biomarker in TMZ-treated GBMs, even in elderly patients (14,15). Between 25 and 50%

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of GBMs are methylated at the *MGMT* promoter; therefore, using *MGMT* methylation status clinically as a predictive biomarker for GBM patients has received increased attention, with the long-term aim to use it as a biomarker to assign alkylating therapy to individual patients. However, a number of studies have reported discordance between *MGMT* methylation status and treatment outcome, and no clear alternative treatment is available for patients with unmethylated *MGMT* tumors, who derive only limited benefit from TMZ (13).

Based on the signatory gene expression profiles using data generated from The Cancer Genome Atlas (TCGA) (16), four distinct molecular subtypes of GBM were described: Classical, mesenchymal, neural and proneural (17). These subtypes have also been associated with characteristic genetic alterations (17). The classical subtype is associated with an astrocytic expression profile, with frequent epidermal growth factor receptor (*EGFR*) amplification, concomitant chromosome 7 amplification and chromosome 10 loss, and focal deletions of 9p encompassing cyclin-dependent kinase inhibitor 2A (*CDKN2A*). Notably, the classical subtype is not associated with *TP53* mutations, which are common in GBMs (17). The mesenchymal subtype is typified by the expression of mesenchymal markers, with frequent deletions or mutations of the neurofibromin 1 (*NFI*) and phosphatase and tensin homolog (*PTEN*) genes. The neural subtype exhibits expression of neuronal markers and displays various mutations and copy number alterations, including amplification of *EGFR* and deletion of *PTEN*. The proneural subtype exhibits an oligodendrocytic expression signature and is characterized by *TP53* mutations. In addition, focal amplifications of the chromosome 4q12 region, which contains the oligodendrocytic development gene α -type platelet-derived growth factor receptor (*PDGFRA*), or mutations of the isocitrate dehydrogenase 1 gene (*IDH1*) are observed. The TCGA group has recently published an updated comprehensive genomic, epigenomic, transcriptomic and proteomic analysis of >500 GBMs (18). Patients were categorized into the four different groups and the overall survival was stratified based on *MGMT* methylation. Notably, the findings indicated that *MGMT* methylation may be a predictive biomarker for response to treatment, but only in the classical subtype of GBM.

In the present study, *MGMT* methylation and somatic mutations were assessed using semiconductor sequencing technology in the same tumor DNA. The cohort included biopsies from 35 GBM patients, who had been treated uniformly with standard concurrent radiotherapy plus TMZ. The association between *MGMT* methylation status/mutation profile and survival was also evaluated.

Materials and methods

Patient samples. Formalin-fixed paraffin-embedded tissues from patients with GBM, which had been obtained during the initial surgical resection, were collected from the Australian Genomics and Clinical Outcomes of Glioma biobank (www.agogbio.org.au). All the patients provided written informed consent and the study was approved by the Institutional Human Research Ethics Committee of South Eastern Sydney Local Health District (SESLD)

Table I. Summary of patient characteristics and treatment results.

Characteristic	Value
Patients, n	35
Age, years	
Median	58
Range	30.5-79.6
Gender, n	
Male	25
Female	10
Overall survival, months	
Median	13
95% CI	9.7-16.3
Time to progression, months	
Median	6
95% CI	4.7-7.3

CI, confidence interval.

Human Research Ethics Committee (HREC), University of New South Wales (Sydney, Australia). Specimens were obtained prior to any chemotherapy or radiotherapy, and the patient information, including age, gender, extent of tumor removal, pathology and treatment, was recorded. Only patients receiving maximal debulking of the tumor (>85%) and undergoing concurrent chemoradiotherapy (60 Gy in 30 fractions with 75 mg/m²/day TMZ) were included in this study. The number of adjuvant cycles of TMZ, determined by clinical variables including toxicity, response to treatment and progression, was noted. Time to progression (TTP; measured from the start of chemoradiotherapy to the time of clinical progression) was determined by the treating oncologist (Professor Anna Nowak).

MGMT promoter methylation. Bisulfite modification prior to CpG pyrosequencing was performed to assess the percentage level of *MGMT* promoter methylation for each tumor specimen, as described previously (19). Chemically methylated or unmethylated universal human genomic DNA (EMD Millipore, Billerica, MA, USA) controls were included with each batch. The CpG pyrosequencing methylation assay was performed using the PyroMarkTM *MGMT* kit (Qiagen, Alameda, CA, USA) on a PSQTM96 MA system (Qiagen) according to the manufacturer's instructions.

Detection of EGFR gene amplification. *EGFR* amplification was recorded in the patient pathology reports by PathWest Laboratory Medicine (Nedlands, Australia) using the Multiplex Ligation-dependent Probe Amplification assay (MLPA; MCR-Holland, Amsterdam, Netherlands), as described previously (20).

Targeted resequencing of DNA. In order to amplify the target regions of 50 oncogenes and tumor suppressor genes that are implicated in solid tumors, amplicon libraries for individual

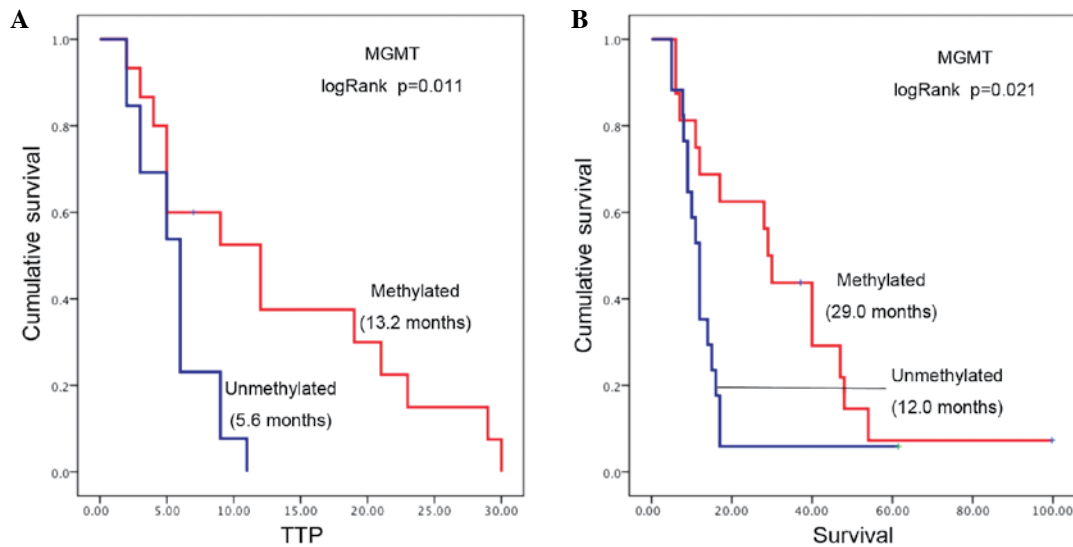


Figure 1. Kaplan-Meier survival curves based on (A) TTP and (B) overall survival for glioblastoma patients stratified by MGMT methylation. Results of log-rank tests and the median survival for each group is provided. MGMT, methylguanine-DNA methyltransferase; TTP, time-to-progression.

OS	5	5	17	62	8	8	9	9	10	11	12	12	12	14	15	16	17	11	12	6	6	7	17	28	29	30	37	40	40	47	48	54	100					
age	59	68	52	62	58	41	36	65	65	50	74	80	66	57	33	51	71	49	50	60	63	69	54	71	55	60	50	38	53	61	51	43	31					
MGMT	[Black bar]																																					
TP53																																						
EGFR																																						
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MPL																																						
MET																																						
SMARCB1																																						
CDKN2A																																						
NOTCH																																						
PIK3CA																																						
RB1																																						

Figure 2. Overall gene alteration patterns for 11 target genes in 33 samples. Blue bars represent single nucleotide polymorphisms, red bars represent amplification, yellow bars represent deletions, grey bars represent MGMT methylation and black bars represent MGMT unmethylation. Numbers inside the bars show the alteration number. Sample number (no.), age (years) and OS (months) are also provided. MGMT, methylguanine-DNA methyltransferase; OS, overall survival.

patient DNA samples were prepared using the Ion AmpliSeq Cancer Hot Spot Panel v2 (Life Technologies, Grand Island, NY, USA), as described previously (21). Subsequently, semi-conductor sequencing was performed using the Ion Personal Genome Machine (PGM) 200 Sequencing kit (Life Technologies) and the Ion PGM Sequencer (21). Torrent Suite™ v3.6.2 software (Life Technologies) was used to parse barcoded reads, align reads to the reference genome (human genome build 19), base call and generate run metrics, including the chip loading efficiency, total read counts, quality and total coverage. In addition, the ANNOVAR™ (Biobase Biological Databases; <http://www.biobase-international.com>) and OncoPrint Gene Browser (Life Technologies) software were used to identify variants, as well as predict amino acid changes and clinical significance. The Integrative Genomics Viewer (Broad Institute, Cambridge, MA, USA; <http://www.broadinstitute.org/igv/>) was used to visualize the alignment of the reads, detect the presence of variants against the reference genome and verify the integrity of variant calls by detecting possible strand bias and sequencing errors (21).

Statistical analysis. The primary end point was the overall survival. A Kaplan-Meier survival analysis was used to generate survival curves and estimates of median survival periods. All statistical analyses were performed using SPSS

software (version 22.0; SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Patient characteristic. A total of 35 patients with a diagnosis of GBM were included in the current study (Table I). The median age was 58 years (range, 31-80 years) and the study included 25 males and 10 females. The median overall survival of the cohort was 13.0 months [95% confidence interval (CI), 9.7-16.3]. The median TTP of the cohort was 6.0 months (95% CI, 4.7-7.3).

The MGMT promoter methylation status was detected in 33 out of the 35 patients, with 16 tumors (48%) found to be methylated at the MGMT promoter region. Tumor MGMT methylation was associated with significantly longer survival rates (median survival, 29.0 months; 95% CI, 20.1-32.9) compared with patients without MGMT methylated tumors (median survival, 12.0 months; 95% CI, 10.1-13.9; log-rank P=0.025; Fig. 1A). A strong association was also observed between MGMT methylation and longer TTP, since the median TTP for patients with methylated MGMT tumors was 13.2 months (95% CI, 4.1-19.9) compared with 5.6 months (95% CI, 4.5-7.5) for patients with an unmethylated tumor MGMT status (log-rank P=0.011; Fig. 1B).

Overall gene alterations. Of the 35 specimens sequenced, multiple (>2) non-synonymous mutations were detected in 10 specimens. In the present study, mutations were identified in the *TP53* (20%), *CDKN2A* (11%) and *PTEN* (9%) genes, as well as mutations and/or amplifications in the *EGFR* gene (29%). In addition, two tumors exhibited mutations in *IDH1*. Mutations in *MPL* (6%), *SMARCB1* (9%), *NOTCH1* (9%), *PIK3CA* (9%), *MET* (6%) and *RBI* (6%) were also detected (Fig. 2).

The mutation profiles were mapped according to *MGMT* promoter methylation (Fig. 2), which revealed that non-synonymous mutations were more frequent in the *MGMT* methylated GBMs compared with the *MGMT* unmethylated GBMs. Furthermore, mutations in the *CDKN2A*, *PTEN* and *PIK3CA* genes were only identified in *MGMT* methylated GBMs. Amplification and/or mutation in the *EGFR* gene and deletion of *CDKN2A* are hallmarks of the classical tumor subtype. A recent study has reported that the predictive value of *MGMT* methylation was only applicable in the classical subtype (15). In the current study, the frequency of *EGFR/CDKN2A* gene aberrations was found to be higher in the *MGMT* methylated group of tumors (56%) when compared with the *MGMT* unmethylated tumors (24%).

Discussion

The presence of *MGMT* promoter methylation in GBM is a predictor of response to TMZ treatment and of overall survival. In the present study, the mutations detected in *MGMT* methylated GBMs were four times higher compared with *MGMT* unmethylated GBMs. This observation may be attributed to a lack of DNA repair function upon presence of lower levels of the *MGMT* protein.

In 2005, *MGMT* promoter methylation was reported to be associated with a survival benefit from alkylating agent chemotherapy in GBM patients, in a companion study based on a phase III concurrent radiation therapy and TMZ trial (13). A number of studies have demonstrated an even higher survival benefit in patients treated with alkylating agents, who presented tumors with *MGMT* promoter methylation, compared with patients having tumors with an unmethylated *MGMT* promoter (13,22,23). Therefore, *MGMT* promoter methylation is a favorable prognostic marker, in addition to a favorable predictive marker for response to TMZ treatment in patients with GBM. The molecular basis for the differential response to TMZ treatment in patients is well established. Alkylating drugs, including TMZ, induce DNA damage by introducing alkyl adducts into the DNA molecules. This causes genetic mutations and cross-links between DNA strands that inhibit DNA replication and thereby trigger cell death. However, *MGMT* removes the alkyl adducts from the DNA as they are introduced, preventing subsequent mutational damage. Tumor cells expressing *MGMT* are thus resistant to alkylating drugs (24).

In the current study, the TTP following chemoradiotherapy and subsequent overall survival were significantly increased in patients with *MGMT* methylation compared with patients without methylated tumors. Thus, the present study confirmed the role of *MGMT* methylation and its associated sensitivity to TMZ (25,26).

The current study investigated the prevalence and types of genomic alterations in GBM and whether these correlate with

MGMT methylation. The results demonstrated that 75% of *MGMT* methylated tumors harbored one or more somatic mutations, compared with 35% of unmethylated cases. Overall, the mutations identified in *MGMT* methylated were four times higher compared with *MGMT* unmethylated tumors. Notably, mutations were identified in the *TP53*, *CDKN2A*, *PTEN* and *PIK3CA* genes in *MGMT* methylated cases. Since these cases also exhibited improved overall survival, further investigations should determine whether these genomic aberrations work synergistically with *MGMT* methylation status and contribute to the response to treatment and overall prognosis. *MGMT* methylation may play a dual role, increasing sensitivity to TMZ and increasing genetic instability, particularly by facilitating the appearance of G to A transition mutations. The TCGA dataset of GBMs revealed increased frequencies of *TP53* and *PTEN* point mutations in *MGMT* methylated GBMs compared with *MGMT* unmethylated GBMs (16). In the current study, mutations in *PTEN* and *TP53* were also found to be associated with *MGMT* promoter methylation, confirming the findings of the TCGA. *MGMT* methylation is commonly associated with G:C to A:T mutations in *KRAS* and *TP53* genes in colorectal cancer (27,28). Therefore, *MGMT* promoter methylation may be an early event in the development of methylated GBM, preceding other characteristic mutations in this subgroup.

In the present study, higher frequencies of *EGFR* and *CDKN2A* alterations were observed in the *MGMT* methylation group. Although the tumors were not categorized into proneural, neural, mesenchymal or classical subtypes (17), the higher frequencies of *EGFR/CDKN2A* indicate that the *MGMT* methylation group may be enriched for the classical subtype. Furthermore, the current study is in accordance with the findings of Brennan *et al* (18), demonstrating that the classical subtype is more responsive to TMZ, which is reflected by the longer TTP and overall survival observed.

In conclusion, *MGMT* methylation was strongly associated with the response to TMZ treatment and overall survival in GBMs, as previously reported. Methylation was also found to be associated with a significantly higher mutation rate. Therefore, it is hypothesized that methylation may be one of the earliest preneoplastic events in this subgroup. A larger cohort of patients is required to further investigate the association of *MGMT* methylation and specific mutations (including those in *EGFR*, *CDKN2A* and *TP53*) with overall survival in TMZ-treated GBMs.

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