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Potential of epigenetic biomarker O6-methylguanine-DNA methyltransferase gene in glioma

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ABSTRACT

Aims: Glioblastomas are the most malignant gliomas in adults with the median survival of 15 months only. O6-methylguanine-DNA methyltransferase (MGMT) is a DNA repair enzyme which overcomes the alkylating chemotherapy effect resulting in chemo-resistance. Methylation at the *MGMT* gene promoter reduces the gene expression and enhances chemo-sensitivity in cancer treatments. Therefore, this study aimed to screen *MGMT* methylation status for a potential epigenetic biomarker in glioma detection and treatmentin glioma patients at Hospital Universiti Sains Malaysia.

Methods: Forty-one glioma paraffin-embedded glioma tissue samples consisting of grade 2 (n=11), 3 (n=10) and 4 (n=20) were analyzed in this retrospective study. The extracted DNA was subjected to bisulfite treatment and the methylation status was determined via methylation-specific polymerase chain reaction targeting the *MGMT* gene promoter.

Results: It was observed that 92.7% of the glioma samples showed methylated and 7.3% unmethylated MGMT promoter. All grade 2 and grade 3 gliomas showed methylation, compared to 85% of grade 4 (p=0.183). More older glioma patients (>40 years) had methylation compared to younger patients (≤40 years) (95.8% vs 88.2%) (p=0.357). More males had methylation compared to females (96% vs 87.5%) (p=0.308).

Conclusions: *MGMT* promoter methylation was found predominant in older (>40 years) male patients with grade 2 and 3 gliomas. High percentages of gliomas, 92.7% harboring methylated *MGMT* promoter, indicate that it is a potential epigenetic biomarker for glioma detection.

Introduction

Glioma is a type of brain tumor that begins in glial cells. It is the most predominant type primary brain tumor which can be found in adults, constituting 30% to 40% of all intracranial tumors, and its utmost prevalence is between the ages of 40 and 65 years (1). In Malaysia, the annual incidence of central nervous system tumors was predicted at 2.8 per 100,000 people by Globocan 2012 (2). Grade 1 tumors are normally benign and can be treated through complete surgical excision. The median survival is 57 months for grade 2 astrocytoma and 24 months for grade 3 astrocytoma (3). About less than 5% of the grade 4

glioblastoma multiforme (GBM) patients were able to live for 5 years after diagnosis (4).

O6-methylguanine methyltransferase (*MGMT*) gene is located at the chromosome 10q26 encoding 207 amino acids that reverse the alkylation effect at the O6 position of guanine (5). Alkylating agents induce methylation at guanine that forms mismatch with thymine which subsequently leads to futile mismatch repair cycle, single-strand DNA breakage and cancer cell apoptosis (6). Removal of O6-methylguanine DNA adduct by the MGMT enzyme prevents the mismatch repair cycle and cancer cell death. Reduction of MGMT protein was able to increase chemosensitivity in high-grade gliomas (7). Another similar study reported that xeroderma pigmentosum fibroblasts transfected with DNA repair protein O6-alkylguanine-DNA alkyltransferase (ATase) were able to rescue the cells from temozolomide (TMZ)-induced damage and lead to resistance (8). Methylation at the *MGMT* promoter region spanning 1.2 kb results in *MGMT* gene silencing (6).

TMZ is an alkylating agent used to treat newly diagnosed and recurrent GBMs. Even with the current advance treatments, patients with stage 4 glioma, GBM, who completed the treatments, have 90% of recurrence rate and recurrent GBMs showed resistance towards previous treatments regimes (9). *MGMT* promoter methylation showed better survival rate in GBM patients with TMZ drug and radiotherapy as compared to radiotherapy only (21.7 vs 15.3 months, p value=0.007) (10). *MGMT* methylated GBM patients were associated with better median progression-free survival and overall survival as compared to *MGMT* unmethylated GBM patients [(8.7 vs 5.7 months, p value <0.0001) and (21.2 vs 14 months, p value <0.0001), respectively] (11).

Failure to identify the specific molecular identity of tumor can result in ineffective treatment and may worsen the prognosis (12). Response to the treatments of glioma patients is mostly dependent on the molecular characteristic of the tumors (13). Although *MGMT* promoter methylation may hold valuable diagnostic and prognostic power in gliomas, the screening test of *MGMT* promoter in gliomas is yet to be introduced in clinical setting due to lack of *MGMT* methylation data in gliomas. Therefore, we sought to determine the *MGMT* promoter methylation status of glioma patients in Hospital Universiti Sains Malaysia, as a potential epigenetic biomarker for diagnostic and treatment stratification to improve the treatment efficacy of current gliomas.

Methods

Tumor Samples

Forty-one archived formalin-fixed paraffin-embedded (FFPE) glioma samples consisting of grade 2 (n=11), grade 3 (n=10) and grade 4 (n=20) were collected from Pathology Department, Hospital Universiti Sains Malaysia. This study is a retrospective study using archived paraffin embedded tissue whereby the protocols received approval by the Human Research Ethics Committee of Universiti Sains Malaysia (JePeM) (ref. no. USM/JEPeM/17050255). Prior to DNA extraction, a neuropathologist reviewed the glioma samples to confirm the tumor types and gradings based on the 2016 World Health Organization criteria (14).

DNA Extraction

Two slices of FFPE glioma blocks, with 5 µm thickness each, were used to extract genomic DNA using GeneJET FFPE DNA Purification kit (Thermo Scientific, USA) according to the manufacturer's instructions. The DNA concentration and purity were determined using NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA) and kept at -20 °C.

Bisulfite DNA Treatment

Commercially available methylated and unmethylated DNA were used as positive controls in the methylation-specific polymerase chain reaction (PCR) (MSP). CpG Methylated HeLa Genomic DNA (New England Biolab, New England) and 5-Aza-dc Treated Jurkat Genomic DNA (New England Biolab, New England) were used as methylated and unmethylated positive controls, respectively. All extracted DNA and controls were subjected to bisulfite treatment using EpiMark Bisulfite Conversion Kit (New England Biolab, New England) according to the manufacturer's instructions. Approximately 10 µL of 100 ng DNA was mixed with 130 µL of bisulfite mixture and subjected to PCR. Thermocycling conditions were 95 °C for 30 seconds, followed by 40 cycles of 95 °C for 15 seconds, annealing for 30 seconds at 50 °C and extension at 68 °C for 1 minute, and finally 68 °C for 5 minutes. This treatment converted unmethylated cytosine nucleotide (C) to uracil (U) whereas methylated cytosine nucleotide (5-mC) remained unchanged.

Methylation-specific PCR

All bisulfite-treated DNAs were subjected to MSP using EpiMark HotStart Taq DNA polymerase (New England Biolab, New England) to determine the methylation status at the *MGMT* promoter. Every treated DNA samples was subjected to two different sets of primers, methylated and unmethylated primers to detect the presence of methylated and unmethylated at the *MGMT* promoter region (15). Methylated primer sequences, forward: 5'- TTTCGACGTTCGTAGGTTTTCGC-3' and reverse: 5'-GCACTCTTCCGAAAACGAAACG-3' amplifies 81 bp PCR amplicon whereas unmethylated primer sequences, forward: 5'-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3' and reverse: 5'-AACTCCACACTCTTCCAAAAACAAAACA-3' amplifies 93 bp PCR amplicon respectively.

PCR amplification was performed in a total of 25 µL reaction containing 100 ng bisulfite-treated DNA, 2.5 µL of 10X EpiMark

HotStart Taq Reaction Buffer, 2 µL of 25 mM $MgCl_2$, 0.5µL of 10 mM dNTP each, 0.5 µL of 10 µM forward and reverse primers and 0.5 µL of EpiMark HotStart Taq DNA Polymerase enzyme. Thermocycling conditions were 95 °C for 5 minutes, followed by 45 cycles of 95 °C for 30 seconds, annealing for 1 minute at 60 °C and extension at 72 °C for 30 seconds, and finally 72 °C for 5 minutes. The PCR products were analyzed using 2% agarose gel.

Normal brain cells were known to harbor unmethylated MGMT promoter only (6). Gliomas samples are often contaminated with normal tissue due to its infiltrative nature (16). Therefore, glioma samples with both methylated and unmethylated *MGMT* promoter were considered as methylated *MGMT* promoter as the unmethylated gene was from the normal brain tissue.

Statistical Analysis

The statistical analysis was carried out using GraphPad Prism software version 5 (GraphPad Software, USA). The association of *MGMT* methylation status with the clinicopathological parameters such as tumor grades, age, gender, and race of the patients was determined using the chi-square test. Statistical significance was defined as $p<0.05$.

Results

The study included 41 glioma samples (age, mean±standard deviation: 42.0±16.8 male sex: 61.0%). All glioma samples were subjected to MSP amplification as shown in Figure 1. Analysis of *MGMT* gene promoter in 41 glioma samples showed either unmethylated only or a mix of both unmethylated and methylated status. Samples with unmethylated *MGMT* promoter only showed a single MSP amplicon of 93 bp. On the other hand, glioma samples with both methylated and unmethylated *MGMT* promoter showed 81 bp (methylated) and 93 bp (unmethylated), respectively.

It was observed that 92.7% of glioma samples showed methylated and 7.3% unmethylated *MGMT* promoter. All grade 2 and grade 3 gliomas showed methylation, compared to 85% of grade 4 (p=0.183). More older glioma patients (>40 years) had methylation compared to younger patients (≤40 years) (95.8% vs 88.2%) (p=0.357). More males had methylation compared to females (96% vs 87.5%) (p=0.308). The correlation of *MGMT* promoter methylation status with the clinicopathological parameters such as tumors grading, age, gender, and the race was shown in Table 1.

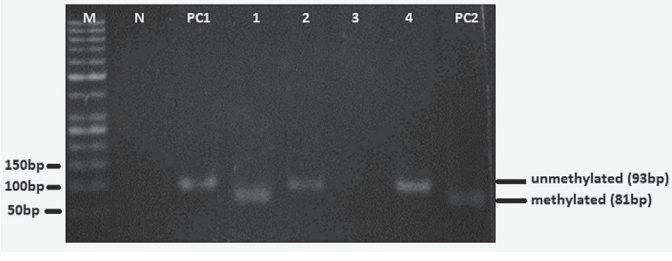

Figure 1. A representative of methylation-specific polymerase chain reaction result of MGMT promoter in gliomas. Lane M: 50 bp DNA ladder, Lane N: Negative control (without DNA), Lane PC1: Positive unmethylated 5-Aza-dc Treated Jurkat Genomic DNA control, Lane 1 and 2: Representative of methylated and unmethylated of a glioma sample, Lane 3 and 4: Representative of unmethylated of a glioma sample, Lane PC2: Positive methylated CpG Methylated HeLa Genomic DNA control

Discussion

According to the Malaysian National Cancer Registry 2007-2011, brain tumors are the second most common cancer among adults and children respectively (17). The current survival rate of stage 4 GBM is still at dismay level. The median overall survival was 6.5 months and median progression-free survival was 5.5 months only (18). Therefore, improving the current cancer therapeutic methods is important to prolong the survival rate. Cancer biomarkers have been extensively studied as it can predict responsiveness to medical treatments, patient's survival rate and disease diagnosis (19). Thus, new biomarker is needed to improve the current diagnosis and treatment stratification.

It was found that reduced MGMT protein expression via promoter methylation showed a significant improvement in the patient's survival rate and responsiveness to alkylating drug treatment (20). GBM patients with *MGMT* methylated undergone radiation showed longer median survival compared to the *MGMT* unmethylated with radiation (15.3 months vs 11.8 months). Together with TMZ and radiation, *MGMT* methylated GBM patients showed much higher median survival compared to *MGMT* unmethylated GBM (23.4 months vs 12.6 months) (21). Hence, *MGMT* promoter methylation status has a high potential for treatment stratification in glioma patients (22-24).

Lack of *MGMT* promoter methylation data in gliomas and ambiguous testing procedure in hospital has impeded the implementation of this potential biomarker in our local clinical setting. Thus, this study was conducted to determine the current methylation status of *MGMT* promoter in our local glioma samples. Besides, this study can contribute to the prevalence of *MGMT* glioma database in Malaysia and globally.

Some glioma samples that showed both methylated and unmethylated *MGMT* promoter were considered as methylated *MGMT* due to the infiltrative nature of gliomas. There is no distinct border between the glioma and normal brain tissue, thus the glioma samples are often contaminated with normal brain tissue. This leads to the presence of normal tissue's DNA in the extracted tumor genetic materials (25). Unmethylated *MGMT* gene in normal tissues produces MGMT protein to repair the damaged DNA (6).

Majority of the glioma samples (92.7%) have methylated *MGMT* promoter. Although there is no significant difference between the tumor grades and *MGMT* promoter methylation

Table 1. Association of MGMT promoter methylation status with tumor grading, age, gender and race among 41 glioma samples

Characteristics	Total no. of samples	Methylation status		p value
		Unmethylated	Methylated	
Number of samples, n (%)	41 (100)	3 (7.3)	38 (92.7)	-
Tumor grading, n (%)				
Grade 2	11 (26.8)	0	11 (100)	0.183
Grade 3	10 (24.4)	0	10 (100)	
Grade 4	20 (48.8)	3 (15)	17 (85)	
Age, years				
Mean±SD	42.0±16.8			
≤40, n (%)	17 (41.5)	2 (11.8)	15 (88.2)	0.357
>40, n (%)	24 (58.5)	1 (4.2)	23 (95.8)	
Gender, n (%)				
Male	25 (61)	1 (4)	24 (96)	0.308
Female	16 (39)	2 (12.5)	14 (87.5)	
Race, n (%)				
Malay	40 (97.6)	3 (7.5)	37 (92.5)	0.229
Chinese	1 (2.4)	0	0	

SD: Standard deviation, MGMT: Methylguanine-DNA methyltransferase

status (p=0.183), we found that all grade 2 and 3 gliomas had methylated *MGMT* promoter compared to grade 4 (85%). On contrary, a study from Europe found that approximately 44.7% of grade 4 gliomas had *MGMT* promoter methylation (10). A study from China showed that 58.6% of GBM showed *MGMT* promoter methylation (26). Based on the GBM group, most of our local sample had *MGMT* promoter methylation (85%) compared to the studies from China (58.6%) and Europe (44.7%).

Approximately 88.2% of younger glioma patients (≤40 years old) exhibited *MGMT* promoter methylation compared to unmethylated *MGMT* (11.8%). About 95.8% of older glioma patients (>40 years old) also exhibited *MGMT* promoter methylation compared to unmethylated *MGMT* (4.2%). Most of the young and old glioma patients showed *MGMT* promoter methylation but slightly more older glioma patients had methylated *MGMT* promoter compared to younger patients (95.8% vs 88.2%). There was no significant association between the patient's age and *MGMT* promoter methylation status (p=0.357). A study showed that more older GBM patients (≥50 years) were found to harbor methylated *MGMT* compared to the younger GBM patients (61.3% vs 38.7%, p=0.444) (26). However, Cancer Genome Atlas project's found that younger patients were associated with glioma CpG island methylation phenotype (G-CIMP) (27).

Our study found that more male patients had methylated *MGMT* promoter as compared to female patients (96% vs 87.5%). However, there is no significance between gender and *MGMT* promoter methylation status (p=0.308). The role of gender in determining glioma prognosis remained ambiguous. The previous study from Italy showed that female GBM patients with methylated *MGMT* promoter were found to have better survival rate compared to methylated *MGMT* males GBM patients (p=0.028) but there was no significance between the unmethylated females and males (p=0.395) (28).

One of the limitations of this study is selection bias. This was because only available FFPE glioma blocks from Pathology Department were selected for this study. This experimental setting was done at the east coast region of Malaysia and the majority race in that population was Malays (29). Thus, this may be explained why most of the samples obtained were from Malays race (97.6%) and only one from the Chinese race (2.4%). Besides, different genetic makeup between Asian and non-Asian may also contribute to the different distribution of *MGMT* promoter methylation in gliomas.

Besides, our small sample size may affect the findings as other glioma studies involve larger sample size such as 169 (28), 206 (10) and 573 (20). As the samples were collected at only one location, the sample size can be improved by collecting from different locations and equal distribution of races in Malaysia to produce more accurate information regarding this potential biomarker.

Moreover, sophisticated equipment can be used to improve the quality of the data in this study as well. Pyrosequencing can detect a very small amount of tumor DNA (5%) in the background of normal DNA. Since glioma is an infiltrative tumor with no distinct border, it would be best to use sophisticated equipment to capture the true tumor genetic nature.

Conclusion

In conclusion, the *MGMT* promoter methylation was predominant in grade 2 and 3 glioma patients, older age (>40 years) and male gender. Nevertheless, this study showed that a high number of glioma samples from Kelantan, Malaysia harbored *MGMT* promoter methylation. Therefore, this gene has great potential in diagnosis and treatment stratification to improve glioma patient's survival rate.

Ethics

Ethics Committee Approval: The study were approved by the Human Research Ethics Committee of Universiti Sains Malaysia (JePeM) (ref. no. USM/JEPeM/17050255).

Informed Consent: Retrospective study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: F.A., Design: Z.I., F.A., Data Collection or Processing: R.M., Analysis or Interpretation: S.S., B.I., Literature Search: W.C.G., Writing: W.C.G., B.I., Z.I.

Conflict of Interest: No conflict of interest was declared by the authors.

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