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I take this chance to welcome your contributions to the IJCBR and have every expectation that it will soon become one of the most respected journals in both the fields of cancer and biomedical research.

Mohamed L. Salem,

Editor in Chief

INTERNATIONAL JOURNAL OF CANCER AND BIOMEDICAL RESEARCH (IJCBR)

RESEARCH ARTICLE

O6-MethylGuanine-DNA Methyltransferase (MGMT) Promoter Methylation Status Analysis in High-Grade Gliomas

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ABSTRACT

Background: The O6-methylguanine- DNA methyltransferase (MGMT) gene is frequently silenced by promoter hypermethylation in malignant gliomas and this has been pinpointed as an epigenetic mechanism reducing MGMT expression levels. The status of MGMT promoter hypermethylation and its relation to tumor progression in gliomas is under extensive study and previous studies have shown conflicting results on the significance of this epigenetic biomarker in relation to the tumor phenotype and clinical outcome. So, in our study, we assessed the role of the epigenetic biomarker; MGMT promoter methylation status, in high-grade glioma patients and correlated the results with the tumor phenotype and clinical outcome. **Methods:** The study included 40 high-grade glioma patients, assessed for MGMT promoter methylation status using methylation-specific PCR (MSP), and correlated the results with clinico-histopathological parameters and survival using appropriate statistical methodologies. **Results:** MGMT promoter methylation analysis revealed 65% of patients with the methylated promoter and 35% with unmethylated ones with no significant prognostic or predictive implications related to different treatment modalities (surgical, chemotherapy or radiation), recurrence rate, or overall survival. **Conclusion:** MGMT promoter methylation status role is not definitive in directing high-grade glioma patients' clinical decision making. Further studies are needed for investigating its role as an epigenetic marker in high-grade gliomas in Egyptian patients.

Keywords: Alkylating agents, DNA Methylation, Epigenetics, Gliomas, MGMT Promoter.

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INTRODUCTION

Central nervous system (CNS) tumors constitute about 3% of all primary malignant tumors and 18% of all malignant tumors (primary and secondary) in Egypt (Ibrahim et al., 2014). Gliomas make up approximately 30% of all CNS tumors and 80% of all malignant brain tumors (Ostrom et al., 2014). Gliomas are defined and graded based on histological features, and pathology is fundamental to predict prognosis and to guide the correct patient management. However, pathological diagnosis can be rather subjective and allows considerable inter-observer variability. Therefore, in a significant number of patients, the histological diagnosis and corresponding expected clinical outcome does not match. Unfortunately, the histological examination does not help distinguishing tumors responding or not responding to the therapy precisely (Goodenberger and Jenkins, 2012).

Recent studies presented molecular genetic analyses as a new approach that could detect subsets of morphologically identical tumors with different clinical behavior (diagnostic markers), describing their prognosis more effectively (prognostic markers). Molecular biological studies may lead to the discovery of gene-based predictors of therapeutic response, helping to guide currently available therapies more rationally (predictive markers) (Wang et al., 2015). In the past two decades, several

biomarkers that provide diagnostic or prognostic/predictive information for malignant gliomas were under continuous study. However, for most of the molecular changes recorded, this does not justify a designation as malignant glioma biomarkers, because biomarkers should provide unique diagnostic, prognostic, or predictive information exceeding that reached by mere histological classification. In this regard, the number of molecular biomarkers in neuro-oncology to date is limited to a few alterations; as O6-methylguanine methyltransferase (MGMT) promoter methylation status (Wang et al., 2015).

The MGMT gene, on chromosome 10 [10q26], is frequently silenced by promoter hypermethylation in diffuse gliomas and thus, has been pinpointed as an epigenetic mechanism reducing MGMT expression levels. The status of MGMT promoter hypermethylation and its relevance to tumor progression in malignant gliomas is currently under extensive study (Möllemann et al., 2005). It is also suggested that it occurs concurrently with hypermethylation of multiple genes and has an association with tumor grade (Dong et al., 2001). Clinical studies have previously demonstrated that MGMT promoter methylation is a positive prognostic marker that renders tumors more sensitive to radiation (Wick et al., 2009). Substantial evidence indicates that the methylation level of MGMT is a positive predictive marker for the responsiveness of newly diagnosed malignant gliomas to alkylation agents (Mur et al., 2015).

Currently, despite the variability of the clinical responses of glioma patients to different treatment modalities, the majority of Egyptian malignant glioma patients especially GBM are presently treated in a uniform standardized way. This standardized way follows a 'one fits all' therapeutic approach, regardless of the individual molecular characteristics of the tumor that most likely affect the patient's prognosis. Consequently, many patients display minor improvement and major therapy-related toxicities. So, the prognostic or predictive value of molecular epigenetic markers is likely to play a significant role in the future clinical management of malignant glioma patients (Dietel et al., 2015). Therefore, we assessed the epigenetic marker, MGMT promoter methylation status, in patients with high-grade glioma. Moreover, we correlated these results with their tumor phenotype and clinical outcome aiming to better classification of Egyptian glioma patients that may help in their clinical decision-making.

METHODS

Study Population and Clinical Characteristics

The study assessed retrospectively forty (40) formalin-fixed paraffin-embedded (FFPE) blocks for patients with malignant gliomas fulfilling the WHO criteria of GBM. The FFPE blocks (9 females and 31 males) were collected from the archives of the Pathology Department, University Hospitals, Egypt, from 2010 to 2013. Inclusion Criteria were: (1) initial pathological diagnosis of WHO grade 3 (analplastic astocytoma and anaplastic oligodendroglioma) or 4 gliomas (GBM) (2) age range from 35-65 years old; (3) both sexes (males and females). Exclusion Criteria were: (1) Patients with other types of malignant tumors or with brain metastasis; (2) Patients with no follow-up records. The clinical data included the patients' medical history and histopathological report. The medical history included: personal history, present history, history, and surgical history; the extent of surgery, post-operative irradiation, chemotherapy, overall survival. The histopathological diagnoses of all specimens were classified according to the WHO classification of tumors of the central nervous system (Louis et al., 2007).

DNA Extraction from FFPE blocks

DNA Extraction was done using QIAamp DNA formalin-fixed paraffin-embedded (FFPE) tissue kit procedure (Qiagen, Germany, Cat no. 56404) to extract DNA from the FFPE blocks (4 sections each 4–5μm thickness) collected in sterile Eppendorf tubes (11). Extracted DNA was subjected to bisulfite treatment using EpiTect Fast DNA Bisulfite Conversion Kit (Qiagen, Germany, Cat no. 59824) was used for this step for efficient conversion and purification of DNA prepared from FFPE specimens, resulting in the conversion of unmethylated cytosine residues into uracil, leaving the methylated cytosines unchanged.

2.1. Methylation Specific PCR

Bisulfite converted DNA was then amplified using methylation-specific PCR (MSP) using HotStarTaq d-Tect Polymerase (Qiagen, Germany). Control reactions were performed with undertaking methylation-specific PCR (MSP) to ensure that the PCR primers are specific for the detection of methylated or unmethylated DNA. For performing control reactions, methylated bisulfite converted DNA, unmethylated bisulfite converted DNA, and genomic DNA was used. 25µl of the EpiTect Master Mix and RNase-free water were dispensed into each PCR with primer solutions and template DNA (<200 ng/50 µl reaction) to each PCR tube. Primers used to detect unmethylated and methylated MGMT sequences, respectively, encompassed: U-MGMT-forward TTTGTGTTTTGATGTTTGTAGGTTTTTGT and U-MGMT-reverse AACTCCACACTCTTCCAAAAACAAAACA (81 bp), M-MGMT-forward TTTCGACGTTCGTAGGTTTTCGC, M-MGMT-reverse GCACTCTTCCGAAAACGAAACG (67 bp). The three steps cycling of the MSP encompassed denaturation for 15s at 94°C, then annealing for 30s at 59.5°C, and extension for 30s at 72°C for 35 cycles. Finally, gel electrophoresis was done using agarose gel 3% concentration and the gel was taken to the UV transilluminator and photographed for documentation and analysis.

In silico data analysis

Genomic sequence data were retrieved from NCBI. Functional and structural analysis of the MGMT gene was performed via ensemble software. Several databases were used for protein analysis (peptide full sequence identification, secondary structure prediction conserved domains, and essential domains identification) including Ensemble, Protein Data Bank, UNiProt/SwissProt, and Potter. Subcellular localization was determined using the compartment program. Protein-protein interaction data was retrieved using STRING database version 10.

Statistical Analysis

Data were analyzed using SPSS for windows version 18 package (IBM Corp., Armonk, NY, USA). Statistical analysis was done guided by the objectives of the study and included appropriate descriptive and analytic statistical methods. Two-sided Chi-square and Fisher's exact tests were used for testing the null hypothesis. Mantel-Haenszel and ANOVA tests were used to estimate the common odds ratio (ORs) and to test whether the overall degree of association is significant. ORs with 95% confidence interval (CI) were calculated. Kaplan Meier curve was used for the association between clinicopathological data of the overall survival and disease-free survival among our study population. The cut-off for statistical significance was $p < 0.05$.

RESULTS

The clinico-pathological findings of the study population

The clinico-pathological findings of malignant glioma patients were summarized in Table 1. Patients were classified into 6 groups according to the tumor site of the glioma. The most prevalent tumor site was in the frontal lobe representing 45% of the study population. GBM was the most prevalent tumor type in our study, representing 82.5% of the study population. All specimens had a higher grade of the tumor; grade III and IV representing 17.5% and 82.5% of the study population. According to the treatment modality of glioma patients, they were classified into two groups with most of the study population was treated by surgical resection of the tumor followed by radiotherapy representing 92.5% of the study population. Regarding the recurrence status of glioma; 80% of patients didn't show recurrence of glioma during their lifetime. Patients were categorized into 3 groups according to their overall survival (OS) and disease-free survival (DFS). The mean OS of the study population was 19.05 ± 8 months. The mean DFS of patients was 16.37 ± 8.3 months, with no significant changes between the OS and DFS results.

Table 1. Clinico-pathological findings of the study population (n=40).

Variable	Number	Percentage
Age		
≥35	6	15%
≥45	22	55%
≥55	12	30%
Sex		
Males	31	77.5%
Females	9	22.5%
Tumor site		
Frontal	18	45%
Temporo-parietal	13	32.5%
Parietal	4	10%
Fronto-parietal	2	5%
Fronto-temporal	2	5%
Corpus Callosum	1	2.5%
Histopathological		
Anaplastic Astrocytoma	4	10.0%
Glioblastoma Multiforme	33	82.5%
Anaplastic	3	7.5%
Pathological Grade		
Grade III	7	17.5%
Grade IV	33	82.5%
Treatment Modalities		
Surgery and	3	7.5%
Surgery and radiation	37	92.5%
Recurrence		
Non-recurrent	32	80.0%
Recurrent	8	20.0%
Disease-free Survival		
≤ 12	8	20%
≤ 24	22	55%
≤ 36	10	25%
Overall Survival (months)		
≤ 12	7	17.5%
≤ 24	23	57.5%
≤ 36	10	25.0%

The incidence map of MGMT Promoter methylation status across the brain revealed a higher incidence of methylated MGMT Promoter (42.5%) in the frontal lobe than those of non-frontal origin. These findings revealed a preferential distribution of MGMT Promoter methylation and implied the distinctiveness among different brain lobes that need further research to know the reason for this preferential distribution.

Description and Analysis of *MGMT* Promoter Methylation Status

Our results for MGMT gene Promoter methylation status showed three variable presentations on gel electrophoresis; where the unmethylated patients are represented as a single visible band in the unmethylated lane only, while methylated cases are demonstrated as a single visible band in the methylated lane or represented as 2 bands in both the methylated and unmethylated lanes (Figure 1). MGMT gene promoter showing methylation status was always accompanied by amplification in the unmethylated reaction as well. This is to be expected since the original tissue sections contained a mixture of tumor and non-malignant tissue. The presence of an unmethylated promoter served as an internal amplification control that could be used to assess the quality and quantity of DNA. Therefore, only tumor specimens that contained a visible methylated signal, with or without an additional unmethylated signal, were interpreted as positive for the MGMT promoter methylation.

In our present study, MGMT Promoter methylation status was successfully determined by MSP in 40 tumor specimens, (26, 65%) showed detectable methylated MGMT promoter, whereas (14, 35%) were unmethylated. For GBM specimens specifically, 63% had a detectable methylated MGMT promoter. The frequency of methylation in both gender (66.7%) methylated female patients *versus* (64.5%) methylated male patients (p=0.617) shows no significant difference with Odds ratio (95% confidence interval) = 0.91 (0.18-4.36).

Association of *MGMT* Promoter Methylation Status and Clinicopathological Findings Characteristics

There is no statistical significance between the methylated and unmethylated results in relation to the clinico-pathological data of patients, demographic, clinical, or pathological characteristics (Table 2).

The median OS among our study population was 17 months with a two-year survival rate of 25%. Methylation status had no impact on OS (p=0.726) nor DFS (p=0.500) **(**Figures 2 and 3).

Structural genomic analysis of *MGMT* Gene

The MGMT gene is located on chromosome 10q26.3 from position 129,467,190 to position 129,770,983 (303794 bases long) (homo sapiens assembly; GRCh38.p2:CM000667.2) (Figure 4).

It is intronless; consisting of six exons. The gene has five transcripts on the forward strand (ENSG00000170430.10). There are 84 regulatory elements located in the region of MGMT. The protein-coding region spans 624 nucleotides; these encoded the 207 amino acid residues with molecular mass 21646 Da. Promoter analysis revealed the presence of GC-boxes at positions -484, -428, -367, and -120.

Structural and functional analysis of *MGMT* protein

The Methylated-DNA--protein-cysteine methyltransferase protein is a single polypeptide chain consisting of 207 amino acid residues with a molecular weight of 21646 Da. It is involved in cellular defense against the biological effects of O6-methylguanine (O6-MeG) and O4-methylthymine (O4-MeT) in DNA repairs the methylated nucleobase in DNA by stoichiometrically transferring the methyl group to a cysteine residue in the enzyme. This is a suicide reaction: the enzyme is irreversibly inactivated; Belongs to the MGMT family. The MGMT protein is predicted to be located in the nucleus (Figure 5). The protein-protein interaction network is depicted in Figure 5 revealed physical and functional associations with other proteins and demonstrated some enriched biological processes which are related to the cellular response to DNA damage stimulus, DNA repair, negative regulation of DNA metabolic process, regulation of DNA metabolic process, and isotype switching.

Table 2. Clinico-pathological findings of patients according to *MGMT* gene promoter methylation status (n=40).

Variables	Unmethylated	Methylated	P-value	OR (95% CI)
Age				
≥35	2 (33.3)	4 (66.7)	0.646	1.0
≥45	9 (40.9)	13 (59.1)		0.72 (0.10-4.82)
≥55	3 (25.0)	9 (75.0)		1.50 (0.17-12.7)
Gender				
Female	3 (33.3)	6 (66.7)	0.306	1.0
Male	11 (35.5)	20 (64.5)		0.90 (0.18-4.36)
Tumor Site				
Frontal	8 (33.3)	10 (66.7)	0.448	1.0
Temporo-parietal	5 (38.5)	8 (61.5)		1.28 (0.29-5.47)
Parietal	0 (0.0)	4 (100.0)		7.28 (0.34-155)
Fronto-parietal	1 (50.0)	1 (50.0)		0.80 (0.04-14.8)
Fronto-temporal	0 (0.0)	2 (100.0)		4.04 (0.17-96.1)
Corpus Callosum	0 (0.0)	1 (100.0)		2.42 (0.08-67.5)
Histopathological Diagnosis				
Anaplastic Astrocytoma	1 (25.0)	3 (75.0)	0.902	1.0
Glioblastoma Multiforme	12 (36.4)	21 (63.6)		0.58 (0.05-6.25)
Anaplastic Oligodendroglioma	1 (33.3)	2 (66.7)		0.66 (0.02-18.1)
Pathological Grade				
Grade III	2 (28.6)	5 (71.4)	0.592	1.0
Grade IV	12 (36.4)	21 (63.6)		0.70 (0.11-4.17)
Treatment Modalities				
Surgical and chemotherapy	1 (33.3)	2 (66.7)	0.724	1.0
Surgical and radiological	13 (35.1)	24 (64.9)		0.92 (0.07-11.1)
Recurrence				
Non-recurrent	12 (37.5)	20 (62.5)	0.507	1.0
Recurrent	2 (25.0)	6 (75.0)		1.80 (0.31-10.3)

Values are presented as number (percentage). A two-sided Chi-square test was used. CI, confidence interval; OR, odds ratio. OR (95% CI) at methylated *vs* unmethylated.

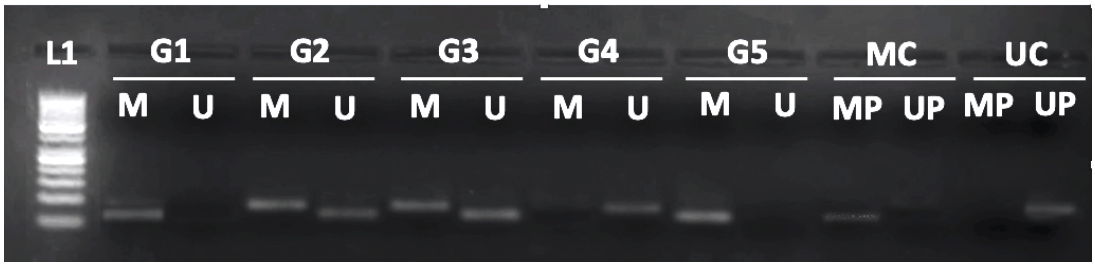

Figure 1. Methylation status of the *MGMT* gene promoter in glioma patients presented on ethidium bromide-stained 3% agarose gel, as determined by Methylation-Specific PCR Assay. Lane 1 (L1): 50bp DNA Ladder. G, glioma; M, methylated, MC, methylated control; MP, methylated primers; U, unmethylated; UC, unmethylated Control; UP, unmethylated primers.

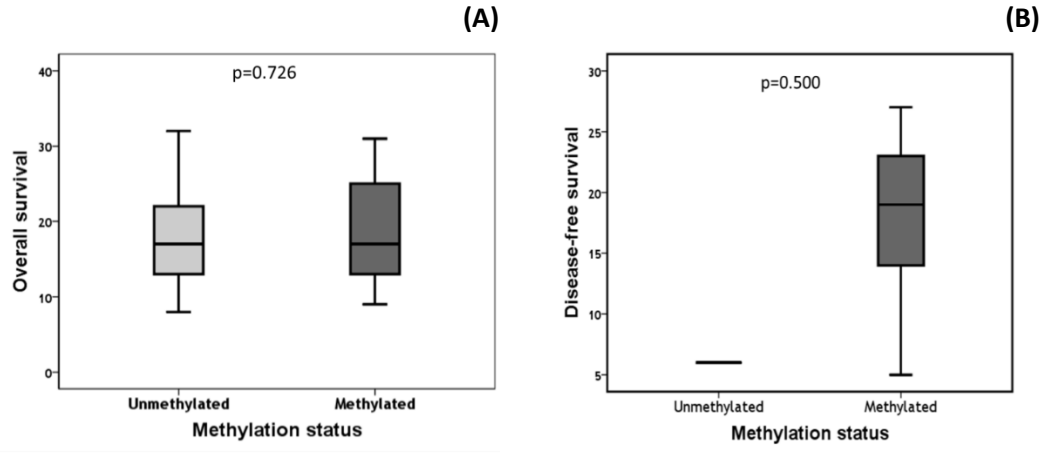

Figure 2. Association between *MGMT* promoter methylation status (A) and overall survival (B) disease-free survival (n=40). Data are presented as box plot (median and quartile).

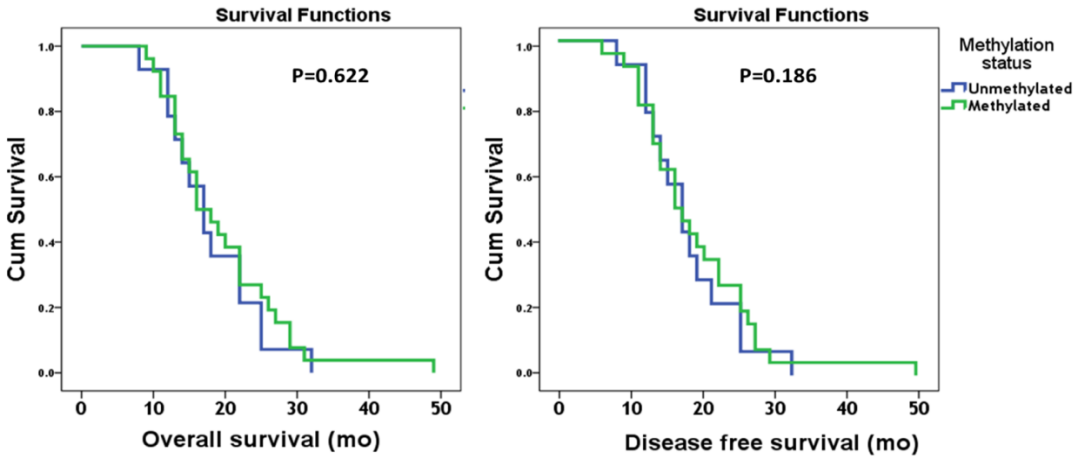

Figure 3. Kaplan Meier curve for the association between *MGMT* methylation status and overall survival and disease-free survival among our study population (n=40). Cum, cumulative; mo, month(s). Log Rank (Mantel-Cox) test was used.

Figure 4. MGMT Gene in genomic location: bands according to Ensembl, locations according to GeneLoc (and/or Entrez Gene and/or Ensembl if different). Genomic Locations for MGMT Gene. chr10:129,467,190-129,770,983(GRCh38/hg38) (https://www.genecards.org

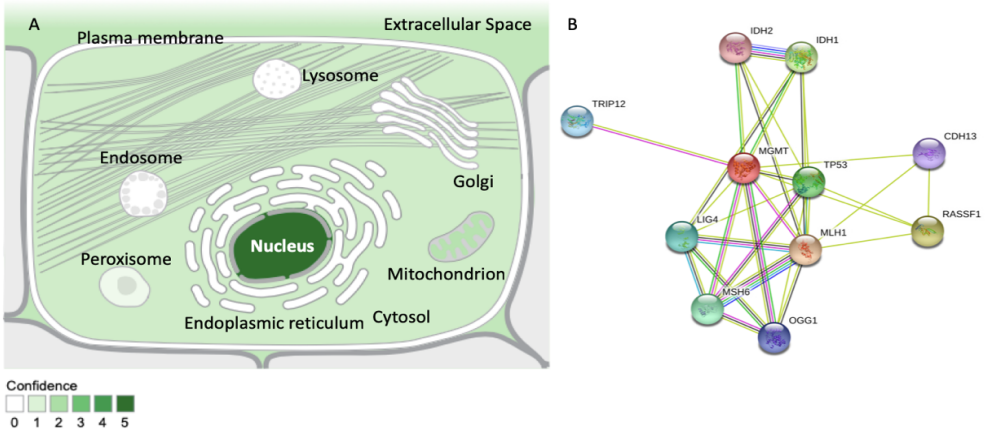

Figure 5. Functional annotation and enrichment analysis of human MGMT protein. (A) Subcellular localization of MGMT protein. The MGMT protein is localized mainly in the nucleus. The confidence of association is noted by the grade of green color with the highest confidence shown by a darker color. The image was derived from Compartments: Subcellular localization database, depending on automatic text mining of the biomedical literature and sequence-based predictions (Data source: Compartment database). (B) Protein-protein interaction (PPI) network. STRING version 10.5. was used to explore known and predicted direct physical and indirect functional associations. The network is composed of 11 nodes and 30 edges, with an average node degree of 5.45 and an average local clustering coefficient of 0.84 (PPI enrichment p-value = 9.9.e- 05). The functional enrichment biological process is represented by node colors (https://www.genecards.org).

DISCUSSION

Generally, promoter methylation is recognized as an important epigenetic mechanism of tumor suppressor gene inactivation during tumor development. Several previous studies have shown that these epigenetic markers can be used as potential therapeutic targets to reverse the methylation (Esteller et al., 2000; Bearzatto et al., 2000; Burgess et al., 2008). Methylation is also known to play an important role in the recurrence of high-grade gliomas (Ma et al., 2013).

Several prognostic markers studied in malignant glioma have given rise to a paradoxical situation (Gömöri et al., 2012), therefore exploring new or validating of existing methylation biomarkers which may help glioma diagnosis, prognosis, or treatment decisions are important (Wager et al., 2008). Thus, understanding the association of promoter methylation status between MGMT across different types of high-grade gliomas and their relevance as to how they could determine tumor progression and influence survival is hence necessary.

According to our knowledge, our study is one of the first to assess the methylation status of MGMT gene promoter status in high-grade glioma Egyptian patients and detecting its correlation with clinic-pathological variables including patients' survival status.

From our work experience, we can deduce that MSP successfully allowed us to assess MGMT promoter methylation status among our study population and archival tissue proved to be adequate for this testing, hence the protocol could be easily incorporated into our routine surgical pathology practice.

Generally, the frequency of MGMT promoter methylation ranges from 30% to 60% in GBM (Majchrzak-Celińska et al., 2015) indicating a slightly higher incidence among our study population than the previously reported findings. However, our results were closely similar to the previously reported frequencies (Hegi et al., 2005; Eoili et al., 2007; Wick et al., 2007; Gorlia et al., 2008; Brandes et al., 2009; Li et al., 2016;).

So, in our study, we attempted to clarify whether MGMT methylation is a biomarker of clinical outcome in high-grade gliomas and if it has a predictive role for therapy or prognostic value for classic clinico-pathological factors to help to solve this treatment decision problem among Egyptian high-grade glioma patients. But our results didn't detect statistically significant correlations, including that for the treatment modality. On the contrary, other researchers (Capper et al., 2008; Weller et al., 2009; Spiegl-Kreinecker et al., 2010) detected a significant role for MGMT Promoter methylation status as

a prognostic and predictive biomarker apparent in the response to chemoradiation using TMZ. This can be explained by the relatively small sample size or due to the presence of heterogeneous groups of patients with different glioma subtypes and who underwent different treatment regimens mainly radiation only after surgery and lack of detailed treatment follow-up history.

The median OS and DFS in our study population are in line with other published studies (Gorlia et al., 2008; Brandes et al., 2009; Brell et al., 2011). These studies also illustrated difficulties in identifying significant determinants of patient survival in relation to MGMT Promoter methylation status. This can be attributed to the relatively small sample size, which may be a limiting factor in achieving statistical significance with a less controlled and more heterogeneous study population than well-designed prospective clinical trials.

Considering the technical part of our study, the method used in our study; MSP method, has been proved to be a sensitive method for assessing MGMT promoter methylation in tumor specimens (Linz et al., 2010); which can be done on FFPE tumor tissues. Our protocol using MSP yielded a good recovery of amplifiable DNA by the commercial DNA Methylation kit (Qiagen, Germany). Despite a recent report arguing in favor of the feasibility and reliability of MSP analysis, suggesting it could be routinely implemented in the clinical setting**,** the use of MSP is often considered not to be so straight forward (Cankovic et al., 2007; Kagan et al., 2007; Shen et al., 2007).

Generally, MSP protocol has technical challenges during the initial validation steps as tissue necrosis, the infiltrating growth pattern of gliomas causing low DNA yields from specimens. In our study population, cutting thicker sections and selecting tissue blocks with the greatest amount of tumor involvement tended to improve the yield of amplifiable DNA. In addition, incorporating positive methylated DNA and negative unmethylated DNA controls in parallel with patient specimens during the bisulfite reaction and PCR amplification steps assured us that optimal conditions were maintained during all testing steps.

CONCLUSIONS

Our study didn't show conclusive prognostic or predictive value for MGMT gene promoter methylation in relation to the clinical and pathological data in high-grade glioma patients. Methylation-specific PCR protocol used in our study to assess MGMT promoter methylation status could be easily incorporated into the routine surgical pathology practice but the results should be interpreted cautiously to help identify glioma patients that may benefit from alkylating agents chemotherapy. Further studies are necessary to replicate and confirm our results, and also to identify the role of MGMT Promoter methylation in disease development and progression.

Abbreviations: MGMT, O6-MethylGuanine-DNA Methyltransferase; MSP, Methylation Specific PCR; CNS, Central nervous system; DFS, disease-free survival; FFPE, formalin-fixed paraffin-embedded; GBM, Glioblastoma Multiforme; TMZ, Temozolomide; OS, overall survival; OR, odds ratio; CI, confidence interval; G, glioma; M, methylated, MC, methylated control; MP, methylated primers; U, unmethylated; UC, unmethylated Control; UP, unmethylated primers.

CONFLICT OF INTEREST

Authors declare that they have no conflicts of interest.

FUDING

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Egyptian Association for Cancer Research (EACR)
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EACR is an NGO society that was declared by the Ministry of Social Solidarity (Egypt) No. 1938 in 19/11/2014 based on the initiative of Prof. Mohamed Labib Salem, the current Chairman of EACR. EACR aims primarily to assist researchers, in particular young researchers in the field of cancer research through workshops, seminars and conferences. Its first international annual conference entitled "Anti-Cancer Drug Discovery" was successfully organized in April 2019 (http://acdd.tanta.edu.eg). Additionally, EACR aims to raise the awareness of the society about the importance of scientific research in the field of cancer research in prediction, early diagnosis and treatment of cancer. EACR is also keen to outreach the scientific community with periodicals and news on cancer research including peer-reviewed scientific journals for the publication of cutting-edge research. The official scientific journal of EACR is "International Journal of Cancer and biomedical Research (IJCBR: https://jcbr.journals.ekb.eg) was successfully issued in 2017 and has been sponsored by the Egyptian Knowledge Bank (EKB: www.ekb.eg).

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