ORIGINAL Article

Prognostic Significance and Clinicopathological Correlations of Epigenetic MGMT Gene Silencing in High Grade Diffuse Gliomas: Experience from a Tertiary Care Centre

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ABSTRACT

Glioblastoma is the most aggressive and commonest primary malignant brain tumour. Current standard of care includes surgery, radiation, and alkylating agent chemotherapy. Despite multimodal treatment, the survival of glioblastoma patients is dismal. Loss of O6-methylguanine-DNA-methyltransferase(MGMT) protein expression due to promoter methylation reduces glioma cell DNA repair activity and resistance to alkylating agents. Thus, in world health organization (WHO) grade 4 diffuse glioma patients treated with an alkylating agent, methylated MGMT promoter is currently being considered a clinically relevant prognostic as well as predictive biomarker. Our aim was to assess the frequency of MGMT promoter methylation in WHO grade 4 diffuse glioma patients and study their prognostic role and clinicopathological correlations.

A two-year prospective cohort research was conducted on 89 WHO grade 4 diffuse glioma patients. The clinical and demographic data were retrieved from our hospital information system. MGMT methylation was assessed using methylation specific polymerase chain reaction. Data was analysed using SPSS-24 software. We studied 89 cases of WHO grade 4 diffuse glioma, of which 38.2% showed methylation of MGMT promoter. There was no significant difference in age, sex, location of tumor and clinical presentation between the methylated and unmethylated groups. A statistically significant association of methylated MGMT promoter was observed with isocitrate dehydrogenase-1 (IDH1) protein expression (p = 0.050) and alpha-thalassemia/mental retardation syndrome X-linked (ATRX) loss (p = 0.003). No significant association was noted with p53 overexpression (p = 0.492) and Ki-67 index (p = 0.698). The median overall survival in these patients receiving standard radiotherapy and concomitant temozolomide chemotherapy showed a trend towards better survival in group with methylated MGMT promoter (p < 0.001).

Our study suggests that methylation of MGMT promoter is more frequent in the subset of grade 4 diffuse gliomas that significantly exhibit IDH1 immunopositivity and loss of ATRX expression. Also, patients who receive radiation therapy and simultaneous temozolomide chemotherapy have a considerably better prognosis and treatment outcome, if the promoter region of MGMT is methylated.

Abbreviations

Isocitrate dehydrogenase 1/2 (IDH1/2); Immunohistochemistry (IHC); Temozolomide (TMZ); O6-methylguanine-DNA-methyltransferase (MGMT); Methylation-specific polymerase chain reaction (MS PCR); Alpha-thalassemia/mental retardation syndrome X-linked (ATRX); Hematoxylin-Eosin (H-E); Polymerase chain reaction (PCR).

Keywords

Alkylating agent, ATRX, Glioblastoma, IDH1, MGMT promoter methylation, TP53, temozolomide.

INTRODUCTION

Glioblastoma (WHO grade 4) is the most aggressive and commonest of all primary malignant brain tumors. The annual incidence is ~3–4 per 100,000 population and is associated with poor prognosis[1]. Assessment of the primary tumor specimens on histology and evaluation of isocitrate dehydrogenase 1/2 (IDH1/2) status on immunohistochemistry (IHC) is currently the gold standard method of diagnosis for all WHO grade 4 diffuse gliomas[2]. In glioblastoma, the survival of patients is dismal despite aggressive treatment protocol, counting for approximately 15-17 months[3]. The treatment protocol currently followed includes surgical resection of primary tumor, followed by radiation therapy, and six cycles of chemotherapy with an alkylating agent like temozolomide (TMZ)[4]. The cellular toxicity of alkylating agents is exerted through alkylation of guanine at its O6 position[5]. O6-methylguanine-DNA-methyltransferase (MGMT), a DNA repair enzyme, rapidly eliminates these alkyl adducts over guanine, and thus counteracts the cytotoxic effect exerted by alkylating agents by preventing the formation of cross-links[6]. This causes resistance to temozolomide[7]. Methylation of MGMT promoter region leads to loss of expression of MGMT protein causing a reduction in the DNA repair activity of glioma cells and thus prevents their resistance to temozolomide[8-10]. The outcome of glioblastoma patients treated with temozolomide is therefore inversely related to the level of expression of enzyme MGMT. Hypermethylation of MGMT promoter highly regulates the expression of MGMT protein and provokes transcriptional silencing[11]. Thus, in WHO grade 4 diffuse glioma patients treated with temozolomide, methylated *MGMT* promoter is now being considered an important and clinically relevant prognostic as well as predictive biomarker[12,13].

Pyrosequencing and methylation-specific polymerase chain reaction (MS PCR) are among the two most popular methods, currently in use to evaluate the methylation status of *MGMT* promoter region. MS PCR is a qualitative technique and utilise both methylation and unmethylation- specific primers. These primers amplify separately fully methylated and unmethylated sequences of *MGMT* promoter respectively[14]. There are a few studies in literature that have identified methylated MGMT promoter as an independent prognostic factor in WHO grade 4 diffuse glioma patients. Hence, evaluation of the methylation status of MGMT promoter region is currently considered mandatory for selection of patients in clinical trials[13,15-17].

Aim of our study was to determine the frequency of MGMT promoter methylation in a cohort of WHO grade 4 diffuse glioma patients and correlate the findings with various clinical, pathological and immunohistochemical markers expression, specifically IDH1, tumor protein 53 (TP53), alpha-thalassemia/mental retardation syndrome X-linked (ATRX), and Ki-67 proliferation index. We have also tried to provide a brief comparison of the findings in our study with those available in the literature.

METHODS

A prospective cohort study was conducted in the Departments of Pathology and Neurosurgery at a tertiary care referral centre for two years, where 89 consecutive cases of WHO grade 4 diffuse gliomas, were included. Patient's demographic and clinical details were retrieved from our hospital information system (HIS) and medical record files. The data included: patient's age at the time of diagnosis, presenting signs and symptoms, site of localization of tumor, imaging findings, extent of surgical resection, type and timing of adjuvant therapy, follow-up time and overall survival in months. Details of the treatment were obtained from all the patients. All these patients were treated with radiation therapy and concomitant chemotherapy with alkylating agent temozolomide (dose: 75 mg/m^2 body surface area daily) after surgical excision of their primary tumor. The tissue retrieved after neurosurgical tumor resection was processed and further analysed by an experienced neuropathologist.

Immunohistochemistry was also carried out in all these cases.

Immunohistochemical procedures
Immunohistochemistry was performed using standard antigen retrieval methods on a 4-µm-thick formalin fixed paraffin embedded tissue sections. The conventional streptavidin biotin peroxidase immunohistochemistry approach was used to evaluate the expression of IDH1 R132H protein, ATRX gene mutation, p53 protein overexpression, and Ki-67 proliferation index. The following primary antibodies were used on one representative block: H09 (mouse monoclonal) clone of IDH-1 antibody (dilution 1:20; category number DIAH09, Dianova), DO7 (rabbit polyclonal) clone of ATRX antibody (dilution 1:200; category number HP A001906, sigma aldrich), Mo a Hu clone of p53 antibody (dilutions 1:100; category number M700101, Dako) and Ki-67 antibody (dilution 1:400; Cell marque). The sections were counterstained with hematoxylin and dehydrated in graded alcohol. The slides were mounted with DPX, covered with a coverslip and evaluated by an independent, experienced neuropathologist.

IDH1 R132H
Positive: Vast majority of tumor cells showed strong cytoplasmic positivity with or without nuclear staining.
Negative: No staining/ weak diffuse staining and staining of macrophages.
The mutation status of IDH1 was determined for all the cases in accordance with the current WHO classification of CNS tumors (2021). Cases were classified into: astrocytoma- IDH mutant (WHO grade 4) and glioblastoma- IDH wildtype (WHO grade 4) depending on their IDH mutation profile.

ATRX
ATRX loss: If the nuclei of tumor cells were negative (unstained) whereas the nuclei of non-neoplastic cells such as endothelia, microglia, reactive astrocytes and lymphocytes showed strong positivity. Loss of nuclear immunostaining for ATRX protein in >10% of tumor cell nuclei was considered as positive for ATRX gene mutation.

Tumor protein 53 (p53)
Overexpression: A minimum of 10% or more of strong positive tumor cell nuclei.

No overexpression: No immunostaining or staining in <10% tumor cell nuclei.

Ki-67 proliferation index was defined as the average percentage of positive nuclei per 1000 nuclei at x400 magnification.

DNA extraction
Areas with high content of tumor cells (70-90%) were selected and dissected out for further analysis. Formalin-fixed paraffin embedded tissue sections were used for the extraction of Genomic DNA using standard Qiagen tissue DNA extraction kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). The extracted DNA was quantified using Nanodrop Lite Spectrophotometer, stored at appropriate temperature and used further for MS PCR.

Analysis of MGMT promoter methylation status by MS PCR
The status of MGMT promoter methylation was assessed by MS PCR. MS PCR is a method of analysing the DNA methylation patterns on CpG islands. For performing MS PCR, bisulfite-modified DNA is amplified using two pairs of primer, which detect methylated and unmethylated DNA, respectively. The protocol includes chemical modification of unmethylated cytosine to uracil, followed by a nested two-stage PCR. Steps of the procedure were as follows:

- Isolated DNA from each sample was subjected to bisufite conversion using Bisulfite conversion kit (EZ Gold DNA methylation kit; M/s. Zymo Research, Orange, CA, USA).
- Polymerase chain reaction (PCR) was performed next using methylation specific primers.
- First stage primer recognized the bisulfite modified template flanking the MGMT gene but did not discriminate between methylated and unmethylated alleles.
- Primer sequences for first stage PCR were:
5#-GGATATGTTGGGATAGTT-3# (forward primer)
5#-CCAAAAACCCCAAACCC-3# (reverse primer)
- For stage 1, the PCR amplification protocol was as follows: an initial denaturation step of 5 min at 94°C; followed by 40 cycles of 30s at 94°C, 30s at 52°C and 30s at 72°C and a final elongation step of 7 min at 72°C. Methylation and unmethylation primers were used separately for each test.

- Primer sequences for unmethylated reaction were:
5#-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3# (forward primer)
5#-AACTCCACACTCTTCCAAAAACAAAACA-3# (reverse primer)
- Primer sequences for methylated reaction were:
5#-TTTCGACGTTCGTAGGTTTTCGC-3# (forward primer)
5#-GCACTCTTCCGAAAACGAAACG-3# (reverse primer).
- For stage 2, the PCR amplification procedure was as follows: an initial denaturation step of 5 min at 94°C, followed by 35 cycles of 15s at 94°C, 15s at 62°C and 15s at 72°C and a final elongation step of 7 min at 72°C.
- MSP was performed to amplify 83 base-pair fragment of the methylated MGMT gene promoter and 91 base-pair fragment unmethylated product.
- Amplified products were then separated on 4% agarose gel, stained with ethidium bromide and visualized under UV illumination.

Statistical analysis
The data was analyzed using SPSS-24 software. Descriptive statistics of continuous variables have been presented in mean ± standard deviation / median (interquartile range) as appropriate. Categorical data has been presented in frequency (%). To compare the median distributions between two groups / three groups, independent t-test / Mann Whitney U test / Kruskal Wallis H test has been used. Chi square test has been used to compare the proportions between groups. In case any cell had an expected count < 5, Fisher's exact test has been used in place of Chi square test. The patients were followed up for a period of three years to determine the overall survival from the day of the diagnosis. Kaplan Meier analysis was used to evaluate the survival outcome probabilities. A *p* value < 0.05 was considered statistically significant.

RESULTS

89 cases of WHO grade 4 diffuse glioma diagnosed on histopathology were included in the study. It comprised of 63 males (M) and 26 females (F) (M: F = 2.4:1). Mean age was 46.78 ± 15.49 years, and the age range was 7-73 years. Demographic and clinical details of the study cohort is presented in **Table 1**.

Table 1. Demographic and clinical profile of the study population

Demographic and clinical characteristics		Number (n)	Percentage (%)
Age (mean ± SD)	46.78±15.49		
Gender	Male	63	70.79
	Female	26	29.21
Site of tumor	Corpus callosum	3	3.37
	Frontal lobe	26	29.21
	Fronto-parietal	2	2.25
	Fronto-temporal	4	4.49
	Insula	5	5.62
	Midbrain and pons	1	1.12
	Occipetal lobe	1	1.12
	Parietal lobe	5	5.62
	Parieto-occipital	6	6.74
	Perisylvian	2	2.25
	Temporal lobe	19	21.35
	Temporo-occipital	2	2.25
	Temporo-parietal	5	5.62
	Thalamus	2	2.25
	Trigone	6	6.74
Clinical presentation	History of loss of consciousness	4	4.49
	Headache	68	76.40
	Vomiting	28	31.46
	Bladder/bowel incontinence	2	2.25
	Impaired vision	13	14.61
	Hemi/ Para / Quadriparesis	26	29.21
	Seizure	22	24.72
	Papilloedema	7	7.87
	Impaired memory	12	13.48
	Ataxia	25	28.09

Immunohistochemistry was applied for IDH1, ATRX, p53 and Ki-67 index in all the cases. 8/89 (9%) cases that showed positive staining for IDH1 on immunohistochemistry were designated as astrocytoma- IDH mutant (WHO grade 4) whereas 81/89 (91%) cases that were negative IDH1 immunostaining were designated as glioblastoma-IDH wildtype (WHO grade 4) in accordance with the current WHO classification of tumors of CNS (2021). Histology of both the groups was similar, showing increased cellularity with moderate to marked nuclear pleomorphism, increased mitotic activity, microvascular proliferation, and/ or necrosis. Loss in expression of ATRX protein was identified in 21.3% (19/89) cases and was more prevalent in WHO grade 4 IDH mutant astrocytomas (7/8; 87.5%) compared to IDH wildtype glioblastomas (12/81; 14.8%). *p*53 protein overexpression was noted in 63/89 (70.8%) cases.

Among WHO grade 4 IDH mutant astrocytomas, p53 protein overexpression was noted in 87.5% (7/8 cases) whereas among IDH wildtype glioblastomas, p53 overexpression was noted in 69.1% (56/81 cases). Majority of grade 4 diffuse glioma patients had a Ki-67 proliferation index of > 10% (93.75%). The immunohistochemical profile of the patient has been presented in **Table 2**.

Table 2. Immunohistochemical profile of the study population

IHC markers		Number (n)	%
IDH	Negative	81	91.01
	Positive	8	8.99
p53	No overexpression	26	29.21
	Overexpression	63	70.79
ATRX	Loss	40	44.94
	Retained	49	55.06
Ki-67	<10%	2	2.25
	>10%	87	97.75

Methylation of MGMT promoter was detected in 38.2% (34/89) cases. The group comprising of IDH-mutant WHO grade 4 astrocytomas frequently showed methylation of MGMT promoter (6/8; 75%). Among glioblastomas- IDH wildtype, methylation of MGMT promoter was observed in 34.6% (28/81) cases. This difference in the methylation profile among two groups was statistically significant ($p = 0.05$). A representative photograph of methylated MGMT promoter is shown in **Figure 1**.
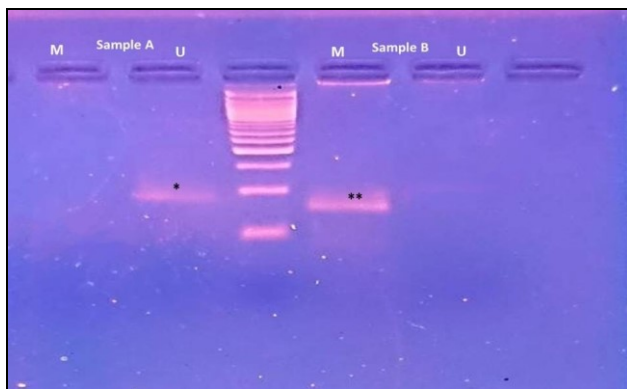

Figure 1. Gel electrophoresis of nested PCR products. **Sample A:** displaying unmethylated **(U)** MGMT promoter (91 base-pair fragment) [indicated by *****] and **Sample B:** displaying methylated (M) MGMT promoter (83 base-pair fragment) [indicated by ******]. The ladder is of 50 base pairs.

Among cases with methylated MGMT promoter, 76.5% were p53 positive (26/34) while among cases with unmethylated MGMT promoter, 67.3% showed p53 positivity (37/55 cases). This difference was not statistically significant ($p = 0.492$) (**Table 3**). There was no significant statistical difference in the Ki-67 proliferation indices between the cases with methylated and unmethylated MGMT promoter ($p = 0.698$) (**Table 3**).

The median overall survival curves for WHO grade 4 diffuse glioma patients receiving standard radiation therapy and concomitant temozolomide chemotherapy showed a trend towards better survival in group with methylated MGMT promoter [29.73 months (SE 1.11)] compared to the group with unmethylated MGMT promoter [10.20 months (SE 0.70)].

This difference was statistically significant ($p < 0.001$). This improved survival in patients with methylated MGMT promoter occurred exclusively at 36 months of follow-up. Also, compared to patients with methylated MGMT promoter, patients with unmethylated MGMT had an increased risk of death at 6 months of follow up. Methylated MGMT promoter was significantly associated with progressively longer survival (**Figure 2**).
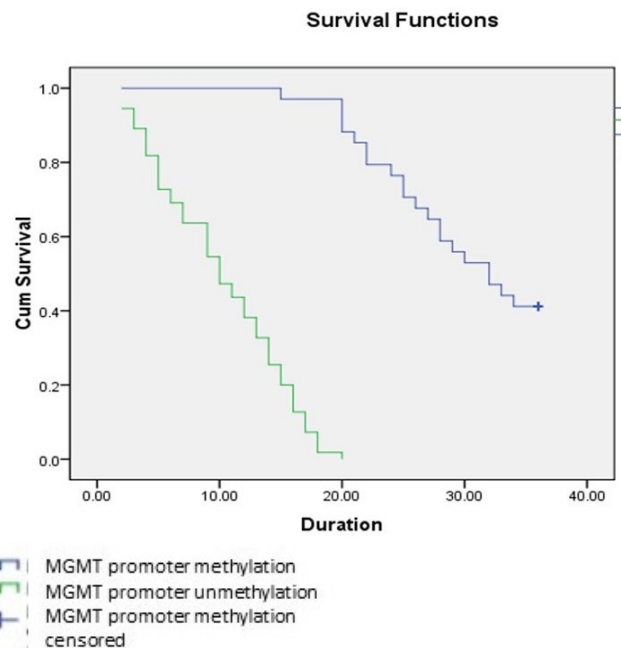

Figure 2. Kaplan Meier curve showing survival outcomes in patients with methylated (presented by blue line) and unmethylated (presented by green line) MGMT tumors.

Table 3. **The clinicopathological profile of MGMT promoter methylated and unmethylated tumors**

Patient characteristics		MGMT promoter methylation				
		Methylated (n=34)		Unmethylated (n=55)		
		N	*%*	*N*	*%*	**p-**value
Age (years)	<18	4	11.76	2	3.64	0.527
	18-30	4	11.76	4	7.27	
	31-40	6	17.65	7	12.73	
	41-50	6	17.65	14	25.45	
	51-60	9	26.47	17	30.91	
	>60	5	14.71	12	21.82	
Gender	Male	25	73.53	38	69.09	0.836
	Female	9	26.47	17	30.91	
Mental Status	Normal	29	85.29	51	92.73	0.442
	Impaired	5	14.71	4	7.27	
Site of tumor	Corpus callosum	2	5.88	5	5.45	0.788
	Frontal lobe	12	35.29	14	25.45	0.346
	Insula	2	5.88	5	9.09	0.788
	Occipetal lobe	0	0.00	2	3.63	0.888
	Parietal lobe	3	8.82	2	3.64	0.366
	Parieto-occipetal	2	5.88	4	7.27	1.00
	Temporal lobe	7	20.59	12	21.82	1.00
	Temporo-parietal	3	8.82	3	5.45	0.366
	Thalamus	1	2.94	3	5.45	0.810
	Trigone	2	5.88	5	9.09	0.788
Clinical signs and symptoms	History of loss of consciousness	1	2.94	3	5.45	0.976
	Headache	24	70.59	44	80.00	0.448
	Vomiting	12	35.29	16	29.09	0.706
	Bladder/bowel incontinence	1	2.94	1	1.82	0.728
	Impaired vision	4	11.76	9	16.36	0.773
	Hemi/ Para / Quadriparesis	14	41.18	12	21.82	0.087
	Seizure	6	17.65	16	29.09	0.336
	Papilledema	2	5.88	5	9.09	0.888
	Impaired memory	2	5.88	10	18.18	0.183
	Ataxia	9	26.47	16	29.09	0.980
IDH1 R132H (8/89)	Mutation present	6	17.65	2	3.64	0.050
p53 (63/89)	Overexpression	26	76.47	37	67.27	0.492
ATRX (19/89)	Loss of ATRX	14	41.2	5	9.1	0.003
Ki-67	<10%	0	0.00	2	3.64	0.698
	>10%	34	100.00	53	96.36	
Survival Status	Alive	14	41.18	0	0.00	<0.001
	Death	20	58.82	55	100.00	

DISCUSSION

CNS gliomas are the commonest primary malignant brain tumor and comprise multiple types in particular astrocytoma, oligodendroglioma, ependymoma and glioblastoma. IDH-wildtype glioblastoma represents the most frequent glioma type with aggressive behaviour and poorer outcome. It has the propensity for widespread invasion including invasion to adjacent tissues[18]. The average median survival of patients with glioblastoma is very less even after aggressive treatment[19,20]. Also,

prognosis of the patients with glioblastoma has remained extremely poor despite multimodal treatment approaches including surgery, radiotherapy and chemotherapy[19]. Methylation of MGMT promoter gene followed by subsequent inactivation of its protein has been substantiated to modulate the response of chemotherapeutic drugs such as temozolomide (an alkylating agent), commonly used in malignant gliomas[18]. Few studies in in literature have authenticated that methylation of MGMT promoter sequence serves as a powerful prognostic and predictive tool for longer overall

survival and progression free survival in patients with glioblastoma [1,13,21].

The frequency rate of methylated MGMT promoter in glioma patients has shown discrepancies across the globe. In literature, the frequency of MGMT promoter methylation by MS PCR varies from as low as 35% to as high as 68%[12,22-28]. In the present study, frequency of MGMT promoter methylation by MS PCR among WHO grade 4 diffuse glioma patients was recorded to be 38.2%. A comparison of the frequency of MGMT promoter methylation in present study with the existing literature has been shown in **Table 4**.

Table 4. A comparison of the frequency of MGMT promoter methylation in present study with the existing literature

S. No.	Author	Number of cases analysed	MGMT promoter methylation (%)
1.	*Jovanovic N et al.* Serbia (2019)[34]	25 glioblastoma	12/25 (48%)
2.	*Johanessen E et al.* Oslo University Hospital, Montebello, Norway (2018)[35]	48 glioblastoma	23/48 (47.9%)
3.	*Arora I et al.* TMH, Mumbai, India (2018)[19]	134 glioblastoma	66/134 (49.1%)
4.	*Uno M et al.* Ludwig Institute for Cancer Research, Brazil (2011)[36]	51 glioblastoma	22/51 (43.1%)
5.	*Jovanovic N et al.* Serbia (2019)[34]	25 glioblastoma	12/25 (48%)
6.	*Nehru GA et al.* CMC, Vellore, India (2012)[20]	27 glioblastoma	17/27 (62%)
7.	*Brandes AA et al.* Italy (2008)[27]	103 glioblastoma	36/103 (35%)
8.	*Eoli M et al.* Milan, Italy (2007)[37]	86 glioblastoma	41/86 (47.7%)
9.	Present study	89 WHO grade 4 diffuse gliomas	34/89 (38.2%) IDH-mutant WHO grade 4 astrocytoma- 6/8(75%) IDH- wildtype glioblastoma - 28/81 (34.6%)

In the current study, no significant difference was observed among the methylated and unmethylated tumors in terms of age ($p = 0.527$) and sex ($p = 0.836$). Similar results were noted by *Arora I et al*[19] in their study on 134 glioblastoma patients. Frontal lobe (35.3%) was the preferred location for tumors with both methylated and unmethylated MGMT promoter. This may be due to the fact that frontal lobe being the largest is also the commonest site for gliomas. This association, however, was not statistically significant ($p = 0.548$). Similar observations were made by *Wang Y et al* [29].

In literature, the association between p53 overexpression and methylated MGMT promoter is highly contradictory. *Shamshara J et al*[30] observed a statistically significant association between p53 mutations and methylated MGMT promoter while *Jesien-Lewandowicz E et al*[31] and *Jha P et al*[32] did not find any association between the two. Current study did not show a significant association between p53 overexpression and methylated MGMT promoter.

Arora I et al[19] observed a statistically significant association between methylated MGMT promoter and IDH1 mutation as well as loss of ATRX protein expression, whereas *Jha P et al*[32] did not find any association between the two. There was a statistically significant association of methylated MGMT promoter tumors with IDH1 mutation ($p = 0.050$) and loss of ATRX protein expression ($p = 0.003$) in the present study. The tumors with methylated MGMT promoter status showed greater frequencies of IDH1 protein expression and loss of ATRX gene mutation compared to the unmethylated tumors.

In the EORTC/NCIC phase III clinical trial, *Hegi ME et al*[33] observed that patients with MGMT methylated tumors live longer, irrespective of the treatment. In their study, the median overall survival of patients with methylated MGMT promoter was 18.2 months compared to only 12.2 months in patients without MGMT promoter methylation. In the same study, a 5-year survival analysis demonstrated MGMT promoter methylation as the strongest predictor of treatment outcome and benefit from alkylating agent chemotherapy. The median overall survival was 29.7 months among patients with methylated MGMT promoter whereas it was only 10.2 months among patients with unmethylated MGMT promoter in our cohort of 89 WHO grade 4 diffuse glioma patients treated with standard of care radiation therapy and temozolomide chemotherapy. First-line single chemotherapeutic agent

'temozolomide' was associated with significantly improved overall survival in these patients. The findings of our results showed that temozolomide is an effective therapy for all WHO grade 4 glioma patients irrespective of their IDH mutation profile and methylated *MGMT* promoter is a good prognostic marker in the setting of treatment with an alkylating agent (temozolomide); and may be predictive of improved overall survival.

CONCLUSION

Our study suggests that methylation of MGMT promoter sequence is seen in greater proportion in WHO grade 4 diffuse gliomas that also express IDH1 immunopositivity and loss of ATRX protein expression. Also, methylation of MGMT promoter is an important prognostic and predictive biomarker of better treatment outcome and is significantly associated with longer overall survival in grade 4 diffuse glioma cases receiving radiation therapy and concomitant alkylating agent chemotherapy. First-line single chemotherapeutic agent 'temozolomide' has been associated with significantly improved survival in these patients if MGMT promoter is methylated. Thus, for a clinician, it is important to confirm the methylation status of MGMT before starting the chemotherapeutic treatment of grade 4 diffuse glioma patients after surgery.

Conflict of Interest

The authors declare no conflicts of interest.

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