

Role of Concurrent Methylation Pattern of *MGMT*, *TP53* and *CDKN2A* Genes in the Prognosis of High Grade Glioma

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Abstract

Objective: High grade glioma (HGG) patients show poor prognosis and survival. Promoter methylation of *MGMT* gene induces altered methylation profile across cell cycle regulating genes. Correlating the promoter methylation status between important tumor suppressor genes could enhance the current understanding of glioma progression.

Methods: We analysed individual and concurrent methylation statuses of three tumor suppressor genes, *MGMT*, *TP53* and *CDKN2A* in different types of HGG and their probable effect on progression free survival. MS-PCR was used to analyse the methylation statuses from 48 HGG tumor samples.

Results: Promoter methylation was observed in 89.5% (43/48) of our cohort in atleast one of the genes, most frequent in *MGMT* (75%), followed by *CDKN2A* (35.4%) and *TP53* (29.2%). *MGMT* methylation and *TP53* unmethylation individually were significant ($p=0.001$ and 0.016) for PFS at 14 months. *MGMT* methylation with therapy (RT/CT+RT) was seen to improve PFS. Concurrent methylation was notably seen in oligodendroglial tumors, the frequency between *MGMT*: *TP53* was 20.83%, *MGMT*: *CDKN2A* (27.1%) and *TP53*:*CDKN2A* (14.6%). Interestingly, concurrent methylation of *MGMT*: *TP53*:*CDKN2A* (12.5%) had better 14 month-PFS proportion (80%).

Conclusion: Two gene concurrent methylation of *MGMT* along with either *TP53* or *CDKN2A* decreased the PFS rate, indicating the negative effect of methylation of *TP53* or *CDKN2A*. However, concurrent methylation of all three genes had better prognosis, but could be mainly due to influence of *MGMT* methylation. This study highlights the importance of assessing concurrent promoter methylation and checking its correlation with survival status among HGG.

Keywords: High grade glioma; CpG promoter methylation; DNA repair; Tumour suppressor genes; *MGMT*; *TP53*; *CDKN2A*; Concurrent methylation

Abbreviations

HGG: High Grade Glioma; TSG: Tumor Suppressor Genes; *MGMT*: O-6-Methylguanine DNA Methyltransferase; *TP53*: Tumor Protein 53; *CDKN2A*: Cyclin Dependent Kinase Inhibitor 2A; AOD: Anaplastic Oligodendroglioma; AOA: Anaplastic Mixed Oligoastrocytoma; AA: Anaplastic Astrocytoma; AE: Anaplastic Ependymomas; GB: Glioblastoma; PFS: Progression Free Survival; OS: Overall Survival

Introduction

Human high grade glioma (HGG) are Grade III and Grade IV tumors (WHO, 2007) [1], that are aggressive and malignant contributing to about 2%-10% of all cancers [2]. These tumors are highly resistant to chemotherapy and radiotherapy, therefore patients with HGG show poor progression free survival (PFS) and overall survival (OS) [3,4]. The current standard of treatment includes, chemotherapy with alkylating agents such as temozolomide (TMZ)

along with radiotherapy [4], but most patients' exhibit rapid disease progression.

Alkylating agents are known to induce reversible DNA damage mediated through epigenetic changes [5]. Epigenetic mechanisms such as CpG island methylation or histone modifications regulate gene expression [6]. Aberrant CpG island promoter methylation silences DNA repair as well as tumor suppressor genes, inducing a CpG island mutator phenotype which leads to progression of glioma [7]. These modifications are widely studied and observed more predominantly in HGG [8]. *MGMT* (O-6-methylguanine-DNA methyltransferase) is the direct DNA repair gene with tumor suppressing function which protects cells against damaging lesions caused by alkylation of DNA at the O-6 position of guanine [9]. *MGMT* transfers the methyl group to an active cysteine within its own sequence and gets inactivated. Promoter methylation has been identified as the key mechanism of *MGMT* gene silencing, it rarely undergoes any other mutation or deletion [10]. *MGMT* promoter methylation is currently used as a prognostic and predictive biomarker in glioblastoma, especially those treated with TMZ [11]. Report suggests median PFS for glioblastoma patients with methylated *MGMT* promoter is 11.8 months, whereas for patients with unmethylated promoter it is 8.3 months [12]. Thus,

MGMT promoter methylation is one among the important biomarkers considered for treatment response and to assess survival status, especially in HGG [13].

Relevance of MGMT methylation mediated gene silencing extends beyond the prediction of chemo-sensitivity and reflects a distinct epigenetic profile [14]. Alterations of the global genome methylation by creation of an irregular methylation pattern (formation of O-6-methylguanine adducts) are observed, especially in cancer cells [15]. Persistence of these adducts causes DNA polymerase to misread the base pairing resulting in accumulation of damages [9]. Increased accumulation of adduct formation in promoter region of cell cycle regulating genes leads to tumorigenesis [16].

Hypermethylation of the promoter regions of tumor suppressor genes (TSGs) has been associated with transcriptional silencing and tumor progression [17]. Tumor protein p53 (TP53), one of the key tumor suppressor genes, is known to promote apoptosis, inhibit cell cycle progression, senescence, differentiation and also accelerates DNA repair [18]. Mutations in TP53 were among the first genetic alterations identified in astrocytic brain tumors [19], but the significance of TP53 as a prognostic factor in GB remains unclear [18]. A possible relationship between MGMT gene expression and TP53 have been suggested previously [11,20]. Several studies have provided conflicting results about the association between the expression of TP53 and MGMT [20,21]. Changes in TP53 non-CpG and CpG dinucleotides could be attributable to silencing of MGMT that allows the persistence of O-6-methylguanine and it being read as an adenine [22]. Contradicting reports state MGMT downregulation is associated with TP53 mutations and, TP53 gene over expression down regulate MGMT and vice versa, stating both a direct as well as indirect correlation [20,23,24]. However, there is little information about concurrent methylation pattern of both TP53 and MGMT in glioma [25].

CDKN2A (Cyclin dependent kinase inhibitor 2A /p16) bears a striking resemblance to the paradigmatic tumor suppressor gene, TP53 [26]. This gene codes for a protein that binds to and inhibits cyclin D kinase (Cdk4 and Cdk6), which phosphorylate serine and threonine residues of the retinoblastoma (Rb) protein [27]. Therefore, the p16 protein inhibits cell cycle progression through G1 to S phase by maintaining the Rb protein in the unphosphorylated state [28]. Inactivation of CDKN2A gene expression by aberrant promoter methylation is believed to play a role in the pathway to tumorigenesis, and is frequently reported in many types of head and neck tumors [27,29]. The present study was carried out to explore and understand the association of individual promoter methylation status, and concurrent methylation pattern between each of the above mentioned genes. Correlation with the progression of glioma and thereby its impact on survival across different histological types of HGG were also analysed.

Patients and Methods

Subjects

Post-surgery excised primary HGG tissues from 48 adult patients with no family history of glioma aged between 18 years-72 years (Male: 38, Female: 10), who underwent surgery in the department of Neurosurgery, NIMHANS, Bangalore during 2011 to 2014, were used in the present study. Pre-operative peripheral blood (internal control) with prior informed consent as per the guidelines of Institution ethical

committee, in accordance with ethical standards was obtained. The resected tumor tissues were bisected; one half was stored in RNAlater (Sigma, Missouri, USA) for further experiments. Another half was fixed in 10% buffered neutral formalin, processed for paraffin section, and was used for histopathological grading, which was carried out at the Department of Neuropathology, NIMHANS.

DNA isolation and quantification

Histologically diagnosed tissues which contained >95% tumor cells were used for the genetic analysis. DNA isolation was carried out for tissue samples using Genomic Prep Cells and Tissue DNA Isolation Kit (illustra™ GE Healthcare UK limited, Buckinghamshire, UK) and Genomic Prep™ Blood DNA Isolation Kit (illustra™ GE Healthcare UK limited, Buckinghamshire, UK) for blood samples. DNA was quantified using Nanodrop ND 2000c (Thermo Scientific, Wilmington, USA), and samples having purity of 1.8–1.99 (A260/280) were selected for the study.

Methylation-specific PCR (MS-PCR)

Bisulphite conversion of DNA (500-1000ng of DNA) for CpG promoter methylation analysis was carried out using EpiTect® Bisulphite kit (Qiagen, Hilden, Germany). Methylation-specific PCR (MS-PCR) was performed on the sodium-bisulphite treated DNA samples to amplify the promoter region of the following genes: MGMT, TP53 and CDKN2A. Two pairs of primers (one for methylated and another for unmethylated sequences) were used for each promoter region as described [8, 25]. MS-PCR was performed under standard conditions using Amplitaq Gold (Applied Biosystems, Foster city, USA) master mix, 35 cycles at annealing temperature of 56°C for MGMT, 55°C for TP53 and 60°C for CDKN2A respectively, primers of 0.2 μM was used for all reactions. For each PCR, bisulphite converted DNA isolated from methylated cell line (U87MG cell line) was used as positive methylation control (cultured in laboratory), and converted DNA obtained from peripheral blood as positive unmethylated control. Nuclease free water was used as negative controls. PCR products were separated on agarose gels and visualized using Syngene G: Box F3 gel documentation system, powered by Genesys (Version 4.0) software (Gurgaon, India). MS-PCR was repeated to confirm the results and ensure they were replicable.

Statistical analysis

Statistical analysis was carried out using MedCalc (version 14.12.0) trial version software. Descriptive statistics was calculated for all variables in the study. Association between categorical variables was analysed using Fisher's exact test. Univariate survival analysis was carried out using Kaplan-Meier survival curve (Log rank test). Multivariate analysis was performed using Cox proportional regression analysis (stepwise). Correlation of Progression free survival (PFS) and Overall survival (OS) at 14 months with different variables were the end-point analysis studied. P value less than 0.05 was considered as significant for all analysis.

Results

Demographic profile

The mean age of our cohort was 41.6 ± 15.4 years. Tumor location was predominant in the frontal lobe (64.5%). Occurrence of grade III tumors were significantly seen in the age group ≤ 40 years, and grade

IV tumors in >40 years (p=0.001). 30 patients from the cohort underwent post surgery adjuvant therapy (Chemotherapy with TMZ+ RT: 21 and only RT: 9). Overall PFS and OS was better for patients with age ≤ 40 irrespective of adjuvant therapy (Table1).

| Variables | No. of patients | MGMT | | TP53 | | CDKN2A | | |
|-------------------------------|-----------------|------|----|------|----|--------|----|----|
| | | M | U | M | U | M | U | |
| Age | | | | | | | | |
| ≤40 years | 25 | 17 | 8 | 8 | 17 | 9 | 16 | |
| >40 years | 23 | 19 | 4 | 6 | 17 | 8 | 15 | |
| Position | | | | | | | | |
| Frontal | 31 | 24 | 7 | 10 | 21 | 13 | 18 | |
| Others | 17 | 12 | 5 | 4 | 13 | 4 | 13 | |
| Grade & type | | | | | | | | |
| III | AOD | 15 | 10 | 5 | 7 | 8 | 8 | 7 |
| | AOA | 11 | 9 | 2 | 3 | 7 | 3 | 8 |
| | AE | 3 | 1 | 2 | 0 | 3 | 0 | 3 |
| | AA | 2 | 2 | 0 | 0 | 3 | 0 | 2 |
| | Total | 31 | 22 | 9 | 10 | 21 | 11 | 20 |
| IV - GB | 17 | 14 | 3 | 4 | 13 | 6 | 11 | |
| Post-surgery treatment | | | | | | | | |
| CT+RT | 21 | 17 | 4 | 6 | 15 | 9 | 12 | |
| RT | 9 | 7 | 2 | 3 | 6 | 1 | 8 | |
| No | 18 | 12 | 6 | 5 | 11 | 7 | 11 | |

Table 1: Patient characteristics (Age, Position, Grade, Histology type, and Treatment information) wise Individual Methylation status of *MGMT*, *TP53* and *CDKN2A*.

Promoter methylation status

MS-PCR of 48 HGG patients revealed methylation of at least one gene in 43 samples (89.5%). Methylation was absent in peripheral blood samples for all three genes (Figure 1.A.B.C). There was no significant correlation between individual promoter methylation status with age of patients, position and type of tumors (Table 2). *MGMT* promoter methylation was observed in 36 samples (75%). Grade wise methylation analysis revealed 22 out of 31 (71%) grade III tumors and 14 out of 17 (82.4%) grade IV tumors to be *MGMT* methylated. *TP53* promoter methylation was seen in 14 samples (29.2%), 10 out of 31 (32.25%) were grade III and 4 out of 17 (23.5%) were grade IV tumors. *CDKN2A* promoter methylation was seen in 17 samples (35.4%). 11 out of 31 (35.5%) were grade III tumors and 6 out of 17 (35.3%) were grade IV tumors.

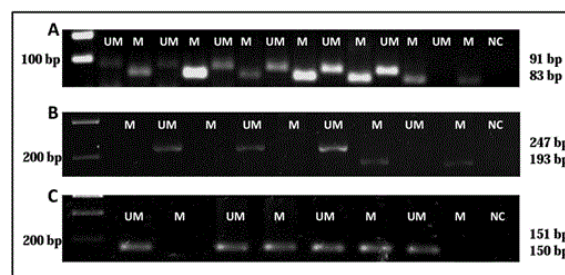


Figure 1

Figure 1: Representative gel images of high grade glioma (AOD and GB) samples. Fig 1A 3% agarose gel shows methylated (83 bp) and unmethylated (91 bp) samples for *MGMT* gene promoter Fig 1B 2.5% agarose gel shows methylated (193 bp) and unmethylated (247 bp) promoter of *TP53* gene Fig 1C 2% agarose gel shows *CDKN2A* gene's methylated (150bp) and unmethylated (151bp) promoters.

Concurrent promoter methylation status between *MGMT*, *TP53* and *CDKN2A* genes

Frequency of concurrent methylation between *MGMT:TP53* is 20.83% (10/48), between *MGMT:CDKN2A* is 27.1% (13/48) and *TP53:CDKN2A* is 14.6% (7/48). Tumor type had an association with the concurrent methylation pattern between *MGMT:TP53* and *MGMT:CDKN2A* genes (Figure 2A & 2B). The distribution of concurrent methylation pattern was similar in *MGMT:TP53* and *MGMT:CDKN2A*, notably seen in oligodendroglial (AOD) type of tumors. Whereas, the concurrent methylation of *TP53:CDKN2A* had a different distribution pattern (Figure 2C). Three gene concurrent methylation (*MGMT:TP53:CDKN2A*) was seen in 12.5% (6/48). Interestingly, concurrent methylation pattern (i.e., either methylated or unmethylated) of these three genes, was seen only in grade III mainly oligodendroglial tumors with significant PFS value (Figure 2D).

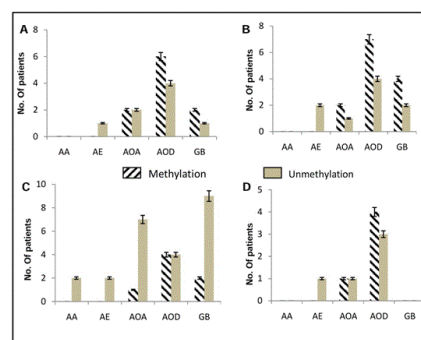


Figure 2

Figure 2: Distribution of two/three gene concurrent methylation pattern among tumor types Fig 2A shows *MGMT-TP53* concurrent methylation status Fig 2B shows concurrent methylation status of *MGMT-CDKN2A* and Fig 2C concurrent methylation status of *TP53-CDKN2A* Fig 2D Concurrent methylation status of all three genes *MGMT*, *TP53* and *CDKN2A*.

| Variables | | No. of Patients | Mean survival (months) | 14 months PFS proportion(%) | Hazard Ratio | P value |
|----------------------------------|-----------|-----------------|------------------------|-----------------------------|--------------|---------|
| Age | ≤40 years | 25 | 11.83(10.10-13.55) | 57 | 1 | 0.187 |
| | >40 years | 23 | 10.22(9.09-12.30) | 34.9 | 1.82 | |
| Grade | III | 31 | 12.88(11.84-13.91) | 71 | 1 | <0.0001 |
| | IV | 17 | 8.2 (6.06-10.36) | 0 | 6.60 | |
| Position | Frontal | 31 | 11.63(10.10-13.15) | 49.9 | 1 | 0.284 |
| | Others | 17 | 10.57(8.68-12.47) | 37.4 | 1.60 | |
| Therapy | CT+RT | 21 | 13.85(13.56-14.13) | 71.1 | 1 | <0.0001 |
| | RT | 9 | 11.22(9.03-13.42) | 61.1 | 2.68 | |
| | No | 18 | 8 (5.94-10.06) | 0 | 7.60 | |
| MGMT Methylation status | | | | | | |
| G-III Methylated | | 22 | 13.62(12.89-14.35) | 83.3 | 1 | 0.02001 |
| Un-methylated | | 9 | 11.11(8.40-13.82) | 44.7 | 5.40 | |
| G-IV Methylated | | 14 | 9.40(7.27-11.53) | 15.7 | 1 | 0.001 |
| Unmethylated | | 3 | 2.67(1.60-3.74) | 0 | 5.96 | |
| TP53 Methylation status | | | | | | |
| G-III Methylated | | 10 | 13.20(11.71-14.68) | 90 | 1 | 0.274 |
| Unmethylated | | 21 | 12.72(11.37-14.07) | 56.8 | 3.03 | |
| G-IV Methylated | | 4 | 4.25(0.83-7.67) | 25 | 3.54 | 0.016 |
| Unmethylated | | 13 | 9.51(7.28-11.75) | 17.9 | 1 | |
| CDKN2A Methylation status | | | | | | |
| G-III Methylated | | 11 | 13.10(11.43-14.77) | 90 | 1 | 0.345 |
| Unmethylated | | 20 | 12.75(11.43-14.07) | 60.7 | 2.65 | |
| G-IV Methylated | | 6 | 8.17(4.76-11.58) | 16.7 | 1.02 | 0.961 |
| Unmethylated | | 11 | 8.18(5.48-10.88) | 35.4 | 1 | |

Table 2: Univariate analysis showing 14 month PFS proportion.

Survival analysis

Follow-up data was obtained from all 48 patients. PFS and OS at 14 months were analysed separately for grade III tumors using univariate analysis (Table 2), as both the grades have different mean/median survival months [30]. Median OS was estimated for grade IV patients. Among the grades and type of tumors, grade III oligodendroglial tumors were seen to have higher PFS and OS. The PFS data obtained for all the variables analysed (Table 2).

Correlation of Methylation status with survival

PFS

Survival analysis was performed to understand the individual methylation status of these three genes (Table 2). Grade III patients with methylated *MGMT* promoter had a mean PFS of 13.62 months (12.89-14.35) and those with unmethylated promoter had mean PFS of 11.11 months (8.40- 13.82) with a significant p value of 0.026. Patients with *TP53* methylation had a mean PFS of 13.20 months (11.71-14.68) and for those with unmethylated promoter, mean PFS was 12.72 months (11.37-14.07). Similarly, patients with *CDKN2A* methylation had mean PFS of 13.10 months (11.43-14.77) and a mean PFS of 12.75 months (11.43-14.07) for those with unmethylated promoter (Table 2).

| Variable | Hazard ratio | 95% C.I. | P value |
|-------------------------|--------------|------------|---------|
| MGMT | | | |
| Methylated | 1 | | 0.12 |
| Unmethylated | 2.12 | 0.84-5.37 | |
| TP53 (Grade IV) | | | |
| Methylated | 1 | | 0.03 |
| Unmethylated | 0.24 | 0.07-0.88 | |
| Adjuvant therapy | | | |
| Yes | 1 | | 0.0001 |
| No | 8.98 | 3.09-26.08 | |

Table 3: Multivariate analysis for Independent Prognostic risk factors.

Grade IV patients with methylated *MGMT* promoter had a mean PFS of 9.40 months (7.27-11.53) and those with unmethylated promoter had mean PFS of 2.67 months (1.60-3.74) with a significant p value of <0.01. Patients with *TP53* methylation had a mean PFS of 4.25 months (0.83-7.67) and for those with unmethylated promoter, mean PFS was 9.51 months (7.28-11.75) with a significant p value of 0.016. Patients with *CDKN2A* methylation had mean PFS of 8.17 months (4.76-11.58) and for those with unmethylated promoter mean PFS was of 8.18 months (5.48-10.88).

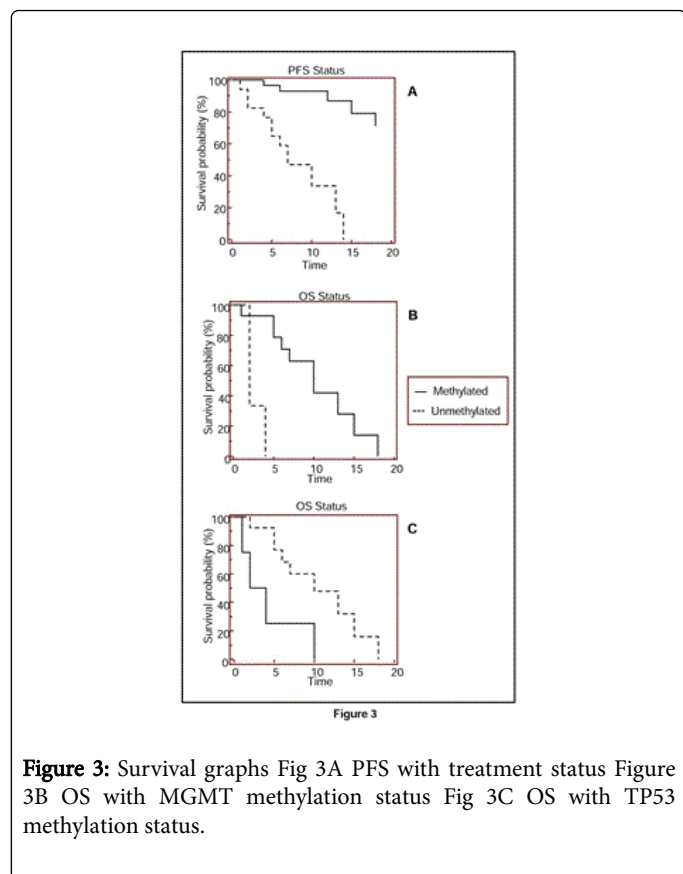
Mean PFS for patients with all three genes concurrent methylation was 13.20 months (11.80-14.60) and for concurrent unmethylation was 10.60 months (6.85-14.34). The details of three and two gene concurrent methylation are given (Table 4).

| Variables | | No. of patients | Mean survival (months) | 14 months PFS proportion (%) | P value |
|---|-----|-----------------|------------------------|------------------------------|---------|
| Concurrent methylation status of all three <i>MGMT</i> , <i>TP53</i> and <i>CDKN2A</i> | MMM | 6 | 13.20(11.80-14.60) | 80 | 0.131 |
| | UUU | 5 | 10.60(6.86-14.34) | 30 | |
| Combinations of prognostic analysis of concurrent <i>MGMT</i> : <i>TP53</i> methylation | M M | 10 | 11.44(8.75-14.13) | 68.6 | 0.172 |
| | U U | 8 | 9.25(5.84-12.66) | 25 | |
| Combinations of prognostic analysis of concurrent <i>MGMT</i> : <i>CDKN2A</i> methylation | M M | 13 | 12.72(11.41-14.04) | 55 | 0.352 |
| | U U | 8 | 10.36(7.02-13.73) | 41.7 | |
| Combinations of prognostic analysis of concurrent <i>TP53</i> : <i>CDKN2A</i> methylation | M M | 7 | 11.88(9.22-14.56) | 68.6 | 0.479 |
| | U U | 24 | 11.79(10.35-13.24) | 60.2 | |

Table 4: Univariate analysis with concurrent methylation.

OS of grade IV tumors

Patients with grade IV tumors had median OS of 10 months (6-13) for *MGMT* methylated and 2 months (2-4) for *MGMT* unmethylated ($p=0.0007$) (Figure 3B). *TP53* methylation had median OS of 3 months (1-10) and for unmethylated 10 months (6-13) ($p=0.016$) (Figure 3C). *CDKN2A* methylated had median OS of 8.5 months (4-12) and for unmethylated it was 10 months (5-13) ($p=0.960$). Univariate analysis of *MGMT* methylation status with therapy showed a significant result ($p<0.0001$).



In multivariate analysis, we observed that *MGMT* unmethylation, *TP53* methylation and no treatment were independent risk factors for faster progression of HGG (Table 3). The role of *MGMT* methylation status along with therapy was analysed to predict the PFS. The hazard ratio obtained was 8.98 (3.09 to 26.08) with a p value <0.0001 , suggesting that *MGMT* unmethylated patients with no adjuvant therapy have a higher risk of progression.

Discussion

Promoter methylation is recognized as an important epigenetic mechanism of TSG inactivation during tumor development. Recent studies have shown that these epigenetic markers can be used as potential therapeutic targets to reverse the methylation [6, 7, 31]. Methylation is also known to play an important role in the recurrence of glioma [32]. Several prognostic markers studied in glioma have given rise to a paradoxical situation [33], therefore exploring new or validation of existing methylation biomarkers which may help glioma diagnosis, prognosis, or treatment decisions are important [8]. Understanding the association of concurrent promoter methylation status' between *MGMT*, *TP53* and *CDKN2A* genes across different types of HGG and their relevance as to how they could determine tumor progression and influence survival is hence necessary. According to our knowledge, this is among the preliminary study to explore concurrence between the promoter methylation of these three genes which individually have important functional role in gliomagenesis.

In our cohort, promoter methylation of *MGMT* was seen in higher frequency, followed by *CDKN2A* and *TP53*. *MGMT* promoter methylation frequency was more in grade IV, in accordance with earlier reports on adult HGG which shows frequency to be between 60-85% [11,34,35]. Promoter methylation of the *MGMT* gene compromises DNA repair and has been associated with a longer PFS in glioma patients [36]. In our study, when *MGMT* methylation was correlated individually with PFS, it was found to be a good prognostic indicator with decreased tumor recurrence and improved OS. *MGMT* promoter methylation leads to reduced gene expression and is known to enhance adjuvant therapy response [10,36]. Similarly our patients with a methylated *MGMT* promoter who underwent adjuvant therapy had a better prognosis and longer survival as reported by other studies [3,37].

Concurrent methylation of *MGMT* with either of the other two genes, showed a similar distribution pattern especially in oligodendroglial tumors (Figure 2A & B), this could be due to the similar biological function of *TP53* and *CDKN2A* in the regulation of cell cycle. Grade III oligodendroglial component tumors were seen to have the highest frequency of concurrent methylation and showed a pattern quite different from other tumor types. The reason being, oligodendroglial tumors are known to have mutations in *IDH1* gene which induces an irregular CpG island methylation across the genome [38]. *MGMT* and *TP53* are known to influence each other functionally; the formation of DNA adducts activate p53 controlled cell cycle as well as *MGMT* mediated DNA repair [11, 20]. There are some reports showing lack of correlation between these two genes [21]. However, our analysis revealed the negative effect of *TP53* methylation on PFS, when concurrent methylation between the *MGMT: TP53* was observed. PFS proportion decreased to 68.6% as compared to PFS rate when *MGMT* was individually methylated (83.3%). A similar association between concurrent methylation of *MGMT: CDKN2A* was observed. This could suggest a possible interdependency of methylation between these three genes.

Three gene concurrent methylation (*MGMT:TP53:CDKN2A*) was seen only in grade III tumors (mainly oligodendroglial) and showed the highest 14-month PFS rate of 80%, whereas concurrent unmethylation had PFS rate of 30%. Oligodendroglial tumors are known to have specific genetic markers not seen in the other histological types [39]. Likewise, concurrent methylation pattern could also be analysed by further studies, to see whether it can be made another signature. This may suggest that the occurrence of three gene concurrent methylation could also serve as a biomarker for analyzing recurrence of HGG. However, *MGMT* methylation among these three could be responsible for better PFS, evidenced by our study and other studies which have shown individual *MGMT* methylation to be a good prognostic indicator. Further, these patients with three gene concurrent methylation had increased PFS which could be attributed to adjuvant therapy (CT with TMZ+RT / RT) that they underwent.

TP53 is reported to have aberrant gene expression due to high mutations occurring in the gene, but its role in prognosis is still not clear between the different levels of expression observed [18]. *TP53* methylation is reported to be more frequent in lower grade than higher grade tumors [25] and less frequent in primary glioma [18]. Our study showed Grade III tumors had slightly higher frequency of individual promoter methylation for *TP53* and *CDKN2A* than grade IV, suggesting *TP53* methylation frequency reduces with increasing grades. This could be due to the difference in characteristics between the grades, i.e., grade III tumors have faster uncontrolled mitosis, whereas grade IV are necrotic [40]. Individual *TP53* methylation was found to be a significant indicator of poor prognosis in grade IV patients evidenced by faster tumor progression (PFS) and decreased OS, since silencing of *TP53* increases mutant phenotype across the genome [41].

CDKN2A is altered (mutations and exon deletions) more frequently in HGG [42, 43], leading to irregular gene expression pattern, resulting in loss of cell cycle function [29]. We had previously reported that *CDKN2A* (p16) shows decreased expression in HGG which may be due to methylation and it's known to be a poor prognostic marker [44]. We observed about 35% *CDKN2A* methylation in both grades, as reported by earlier studies [45]. However, *CDKN2A* methylation individually did not show any such prognostic association in HGG. Concurrent methylation pattern of *TP53:CDKN2A* was distributed

differently from *MGMT:TP53* and *MGMT:CDKN2A* (Figure 1C), indicating related cell cycle function between *TP53* and *CDKN2A*. Analysis among these three genes, suggest that *CDKN2A* methylation has no significant influence on the methylation pattern of other two genes, as suggested by few other studies [10,46].

PFS and OS depend on many factors like, age, extent of resection, histology type, gene mutations, treatment, treatment response, etc. Studies have shown that treatment response is dependent on methylation status of some important genes [33, 47]. Summarizing, the current study revealed that gene silencing mediated by promoter methylation of TSG is a relatively common event in HGG and could be used as biomarkers. Data indicated *MGMT* methylation with or without adjuvant therapy, in both grades of HGG and *TP53* unmethylation in grade IV has significant correlation with improved prognosis. Oligodendroglial tumors have higher frequency of concurrent methylation of these three genes and were found to have predictive significance, indicated by increased PFS proportion. It will be interesting to study global methylation pattern and status of other important altered genes like *PTEN*, *DMBT1*, *RASSF1A*, *hMLH1*, in the disease progression of HGG.

Conclusion

Exploring the individual and concurrent methylation panel of genes functioning in different pathways leading to gliomagenesis could add further knowledge in this direction. Our study conceptualises the importance of concurrent methylation pattern of these key genes and its probable role in progression of glioma, which could help in clinical decision making and patient management. Genomic studies which involve quantitative promoter methylation analysis/ bisulphite sequencing/microarray analysis correlated with mRNA expression would give improved understanding of the role of concurrent methylation of important genes.

Authors Contributions

JMM carried out the sample collection, genetic studies, conception and design, acquisition of data, interpretation of results, drafting and revising the manuscript. DG carried out the sample collection, genetic studies, conception and design, acquisition and analysing of data and drafting part of manuscript. CGK conceived of the study, and participated in its design coordination and helped to draft the manuscript, agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. NRKVL, DIB and SBMM have given final approval of the version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. SMK, VHN, LCH and AS participated in the design of the study and performed the statistical analysis.

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