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Co-deletion of 1p/19q is Strongly Correlated with a High Level of MGMT Promoter Methylation in High Grade Gliomas as Revealed by Pyrosequencing

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Abstract

Background: MGMT methylation, along with 1p/19q co-deletion and IDH1 mutation, is an important biomarker in high grade gliomas. MGMT methylation indicates an improved response to temozolomide chemotherapy; patients with 1p/19q co-deleted anaplastic oligodendrogliomas benefit preferentially from adjuvant chemotherapy. Pyrosequencing is a method that allows the level of MGMT methylation to be measured in a quantitative manner.

Aim: To compare the mean MGMT promoter methylation level of high grade gliomas and correlate it with other clinical parameters and markers including 1p/19q co-deletion and mutation to IDH1 or IDH2.

Methods: Pyrosequencing was used to quantitatively detect the level of MGMT promoter methylation for 171 high grade gliomas, mutations to IDH1 and IDH2 genes were also detected by pyrosequencing, or immunohistochemistry (n=166). Screening for 1p/19q deletion was by fluorescence in situ hybridisation (n=46). Statistical analysis was performed using R-Stats v2.15.2.

Results: Higher methylation was correlated with lower grade and mutation to either IDH1 or IDH2 (27.0% vs. 16.6% p = 0.008; and 27.5 vs. 16.1 p = 0.002 respectively). 1p/19q co-deletion versus non co-deletion was associated with a particularly high level of methylation (42.2% vs. 17.7% p = 0.001). No significant differences were seen for age or gender.

Conclusions: The results offer a potential explanation for the improved prognosis seen in glioma patients with 1p/19q co-deletion.

Keywords: Pyrosequencing; Glioma patients; Glioblastomas

Background

The gene promoter methylation status of O6-methylguanine-DNA-methyltransferase (MGMT) is now established as an important biomarker in clinical practice for the treatment and management of high grade gliomas [1]. In glioblastomas (GBM; WHO grade IV) MGMT methylation has been shown to be predictive of an improved response to alkylating chemotherapy [2-4]. In addition to the predictive effect conferred by MGMT methylation an improved prognosis has been observed for anaplastic gliomas (WHO grade III) [5-8]. Mutations to IDH1 and IDH2 along with the co-deletion of chromosome arms 1p and 19q are other important glioma biomarkers aiding diagnosis and conferring an improved prognosis [9]. All three biomarkers are highly correlated in the lower grade gliomas (II-III). In primary grade IV gliomas the scarcity of mutations to IDH1 and IDH2 (5-10%), and 1p/19q co-deletions (<5%) compared to the frequency of MGMT methylation (approximately 44%) means that there is much less interdependence between the markers in GBM [9,10].

Formerly methylation-specific PCR was the gold standard for MGMT methylation analysis, partly because of this and for ease of interpretation, MGMT methylation has been treated as a dichotomous variable. Recently pyrosequencing, a quantitative method has come to the fore owing to its reproducibility and sensitivity [11], this allows MGMT methylation to be treated as a continuous variable.

Pyrosequencing is a 'sequencing by synthesis' technique that relies on the measurement of pyrophosphate (PPi) released when the correct nucleotide is incorporated by DNA polymerase, having been dispensed in a predetermined order. ATP sulfurylase converts PPi to ATP which in turn activates luciferase to produce quantifiable light [12]. The MGMT methylation assay requires the bisulphite treatment of the sample DNA. Bisulphite treatment converts Cytosine (C) into Uracil (U), a normal PCR will then change the U back into Thymine (T); in effect Cs are changed to Ts. Epigenetic techniques rely on the fact that methylated Cs are protected from bisulphite conversion and remain as Cs [13]. The methylation level is then determined by the ratio of Cs to Ts at specific CpG islands within the MGMT promoter.

Here we explore whether, similar to the effect on the frequency of methylation, the diagnosis, grade, and the presence or absence of the other biomarkers (namely IDH1 and 2 mutations and 1p/19q co-deletion) has any influence on the mean MGMT methylation, as measured by pyrosequencing in our institution.

Methods

All tumours were diagnosed by consultant neuropathologists (S.A-S, I.B, A.K) as part of the routine diagnostic clinical neuropathology service with full knowledge of clinical, immunological, molecular, and radiological information. The cases were seen in the department between late 2012 and early 2014. A small proportion (≈12%) of cases were from older biopsies (the oldest from 2003) as part of routine neuro-oncology follow up, the molecular biomarkers being processed as they were not available in the department on initial presentation.

For 171 high grade gliomas, seen at our institution, MGMT methylation analysis was performed by pyrosequencing using the CE-marked therascreen MGMT pyro kit (Qiagen) on a Q24 pyrosequencer (Qiagen). The bisulphite converted MGMT promoter sequence containing the four CpG islands assayed was as follows: YGAYGTTYGTAGGTTTTYGT (Y indicates the C or T of the CpG island; dependent on the methylation status). Mutations to IDH1 and IDH2 genes were also detected by pyrosequencing using Pyromark Gold reagents (Qiagen) with protocols adapted from the literature [14,15], or by immunohistochemistry in the manner set out by Capper and colleagues [16,17] (n=166).

DNA was extracted from FFPE specimens and bisulphite converted using the QIAmp DNA FFPE tissue kit and the Epitect Bisulfite kit respectively (Qiagen). Screening for 1p/19q deletion was by fluorescence in situ hybridisation (n=46) as described previously [18].

Statistical analysis was performed using R-Stats v2.15.2 [19]; analyses included t-test, 1-way ANOVA [20] and the Fisher exact test [21], and all p values are 2-tailed. All error bars represent standard error of the mean. MGMT methylation was treated as a continuous variable (mean methylation of the four CpG islands).

Results

The series consisted of 142 WHO grade IV gliomas and 29 WHO grade III gliomas. The grade III gliomas were anaplastic astrocytoma (AA, n=9), anaplastic oligoastrocytoma (OA, n=3) and anaplastic oligodendroglioma (AO, n=17) (Table 1). There were 63 females and 108 males. The median age at presentation was 55 years, ranging from 17 to 78 years. More detailed clinical information can be found in Supplementary Table 1.

Diagnosis	n	IDH mutated	Co-deletion	Methylated‡	Mean Age
GBM	142	8 (5.7)	2 (9.1)	76 (54.3)	56.2
AA	9	6 (75)	0/5	9 (100)	42.8
OA	3	2/2	0/2	3 (100)	46.0
AO	17	15 (100)	9 (56.2.8)	16 (94.1)	43.4

‡ Methylation ≥ 5% (according to manufacturer's instructions).

(%); abbreviations: GBM – glioblastoma, AA – anaplastic astrocytoma, OA - oligoastrocytoma, AO – anaplastic oligodendroglioma, IDH - isocitrate dehydrogenase 1 or 2.

Table 1: Distribution of biomarkers and age by diagnosis

Mean methylation was significantly higher in WHO grade III tumours compared to grade IV (27.0% vs. 16.6% p = 0.008) (Figure 1).

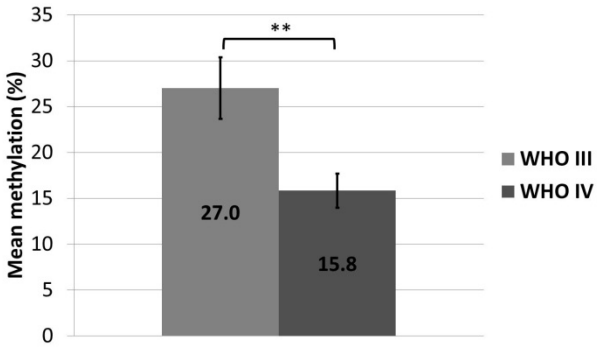

Figure 1: Difference in mean MGMT promoter methylation between WHO grades III & IV. WHO grade III gliomas show a significantly higher mean methylation than WHO grade IV gliomas (** = p≤0.01). Error bars represent standard error of the mean.

In keeping with the figures seen with the WHO grading, 1-way ANOVA revealed there to be significant differences between the gliomas by diagnosis with the highest methylation observed for AO (AO 31.9%, OA 14.1%, AA 22.2% GBM 16.6% p=0.025) (Figure 2). Pairwise analysis showed this to be significant only between AO and GBM.

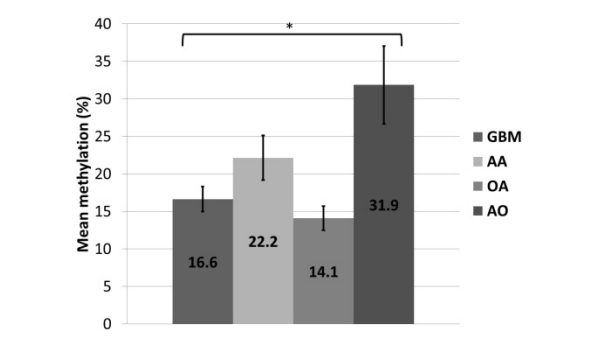

Figure 2: Difference in mean MGMT promoter methylation by diagnosis. The highest mean methylation was in anaplastic oligodendrogliomas. 1-way ANOVA shows there to be significant differences according to diagnosis (* = p≤0.05). Pairwise analysis revealed that the only significant difference was between anaplastic oligodendrogliomas and glioblastomas (* = p≤0.05). GBM – glioblastoma, AA – anaplastic astrocytoma, OA - oligoastrocytoma, AO – anaplastic oligodendroglioma. Error bars represent standard error of the mean.

Reflecting the relative distribution of mutations to IDH1 or IDH2 between WHO grades the difference for mutated and non-mutated

gliomas was almost exactly the same as that between the grades, mutation conferring the higher methylation level (27.5% vs. 16.1% p=0.002) (Figure 3).

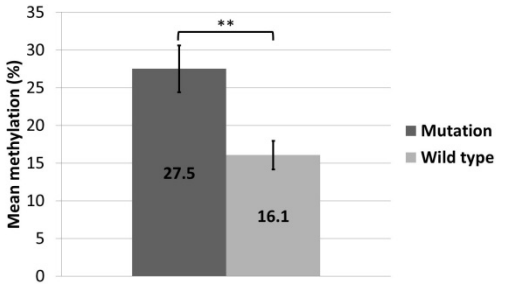

Figure 3: Difference in mean MGMT methylation between gliomas with mutated IDH1 or IDH2 versus gliomas with IDH1 and IDH2 wild type. Mutated gliomas show a significantly higher mean methylation than non-mutated gliomas (** = p≤0.01, n=166). Error bars represent standard error of the mean.

The most interesting observation was that 1p/19q co-deletion versus non co-deletion was associated with a particularly high level of methylation (42.2% vs. 17.7% p=0.001) (Figure 4). The relationship remained significant even in the subset positive for IDH mutation (44% vs. 21% p=0.002, n=24). Pairwise comparisons of 1p/19q deletion status, including singular 19q deletion and no deletion, did not reveal any significant differences in methylation, except with co-deletion.

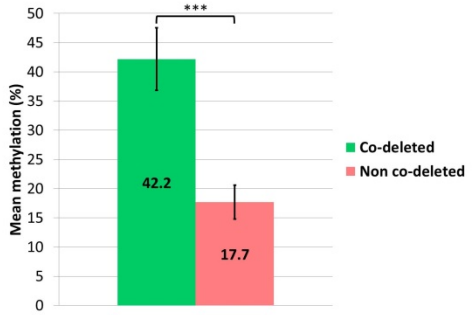

Figure 4: Difference in mean MGMT methylation between gliomas with 1p/19q co-deletion versus those without co-deletion (n=46). 1p/19q co-deleted gliomas were associated with a particularly high level of mean methylation, significantly higher than gliomas without 1p/19q co-deletion (*** = p≤0.001, n=46). Error bars represent standard error of the mean.

There was no association between mean methylation and gender, although it was slightly higher for female patients (20.2% vs. 17.3%, p=0.4). Linear regression did not show any direct relationship between age and mean methylation (adjusted R^2=0.002, p=0.2). This is in contrast to the relationship between IDH mutation and younger age (40.7 years vs. 57.5 years p=1.5E-10).

Discussion

It is already well established that MGMT methylation is strongly correlated with other important glioma biomarkers, mutations to the IDH genes and 1p/19q co-deletion, particularly in WHO grade II and III [5,22]. In our series these correlations remained in place, as can be seen in supplemental table 1; however the purpose of our study was to assess whether this correlation was reflected in the level of MGMT methylation as measured by pyrosequencing. Indeed we found that the mutations to either IDH1 or IDH2 were associated with a higher level of methylation, also probably accounting for the difference seen between WHO grades III and IV, owing to the relative frequencies of mutations in the respective grades [9], as recently shown in a Finnish study [23]. This result was wholly expected, there being a biological mechanism whereby the neo-enzymatic activity of IDH mutants causes a build-up of the oncometabolite, 2-hydroxyglutarate, which inhibits TET2 and histone demethylation resulting in a CpG island methylator phenotype ($CIMP^+$) [24-26]. Less expected and of interest was the very high levels of methylation associated with 1p/19q co-deletion. All but one of the co-deleted tumours carried the R132H IDH1 mutation suggesting a cumulative effect; in fact the one non-mutated tumour had a markedly lower methylation level. Additionally a technical issue of 1p/19q analysis by FISH is that it can sometimes return deletions that do not represent true whole arm deletions (which confer the favourable prognosis) [27]. The presence of a high level MGMT hypermethylation may aid interpretation in these cases.

The validity of the CpG islands used in this study with reference to survival was recently established in a study of primary GBM. The CpG islands (referred to as 76-79 in their study) had clinically significant mean methylation cut-off points for both overall survival (8% HR 0.35, p = 1.53E-04) and progression free survival (9% HR 0.32, p = 2.3E-05) [28]. However the tumours involved in our study were, for the majority, recent presentations with little follow up time so it is not within scope of this study to analyse survival. Given the favourable prognosis associated with all three of the biomarkers tested here one would expect that patients with tumours exhibiting high levels of MGMT methylation, IDH mutation and 1p/19q co-deletion to have a survival advantage over those that lack the full complement. Further analysis of the recent literature reveals that these IDH mutated and 1p/19q co-deleted tumours probably form part of the co-deleted ($CD\text{-}CIMP^+$) phenotype identified by Mur et al, which were shown to have improved survival over normal $CIMP^+$ tumours and CIMP- tumours [29].

In conclusion our results are suggestive of a role for high level MGMT hypermethylation in the improved prognosis seen in glioma patients with 1p/19q co-deletion, and support pyrosequencing as a robust method of analysis that provides extra clinically relevant information.

Authors' Contributions

RL performed the experiments, statistics and wrote the manuscript. LD performed experiments and reviewed the manuscript. MA, IB, AK, and SA-S diagnosed the cases and reviewed the manuscript. CC, RB, RB, LB, and KA provided the cases, patient data and reviewed the manuscript.

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