Quantitative analysis of MGMT promoter methylation status changes by pyrosequencing in recurrent glioblastoma

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Abstract

Background

MGMT promoter methylation status can change in response to several factors, including treatment with alkylating agents. Some authors have attempted to quantify these alterations with inconsistent results. This study aims to determine changes in MGMT promoter methylation status by pyrosequencing, which quantitatively yields results, providing percentages of methylation of a given CpG dinucleotide in a cohort of patients reoperated for recurrent glioblastoma and having previously completed the Stupp protocol.

Methods

A total of 25 pairs of glioblastoma preselected tumor samples were retrospectively analyzed using conventional DNA extraction techniques, bisulfite conversion, and PCR amplification of the MGMT promoter gene. Methylation status was obtained using pyrosequencing of 4 CpG dinucleotides within the enhancer region of the MGMT promoter gene and depicted as percentages or categories (hypermethylated, intermediate methylation, unmethylated). Matched samples were compared using Wilcoxon signed-rank test, and the log-rank test was employed to establish a correlation between survival data and methylation status.

Results

Median value of MGMT promoter methylation status declined after adjuvant treatment from 22.25–16.15%. ($p = 0.872$). A correlation without statistical significance between methylation in primary samples and OS was found ($p = 0.102$). Lower degrees of association were obtained when studying primary methylation and PFS ($p = 0.187$). Intermediate methylation status at recurrence was more linked to PPS ($p = 0.03$)

Conclusions

Switching in both directions was observed when analyzing methylation status as a continuous variable. These data suggest that the dynamics of epigenetics may be very complex and not entirely explained by clonal selection or tumor heterogeneity.

Background

Gene expression can be controlled by promoter regions located in close vicinity to sites where transcription of DNA into RNA begins. Some of these genomic areas are characterized by clusters of cytosine and guanine dinucleotides, called CpG islands. It is well known that hypermethylation of these

CpG islands, catalyzed by enzymes called DNA methyltransferases, can hinder the codification of DNA into proteins without altering nucleotide sequence, leading to an epigenetic silencing (1).

Methylation of the promoter region of the DNA repair enzyme O^6-methylguanine-DNA-methyltransferase gene (MGMT) has been independently implicated in high-grade glioma prognosis and sensitivity to alkylating agents, such as temozolomide (TMZ) for more than a decade (2). MGMT enzyme prevents the process of programmed cell death by removing alkyl groups from guanine residues (3), which, if left unrestored, inevitably lead to futile attempts of repair and eventually the apoptotic cascade.

Most alkylating agents, including temozolomide (TMZ), utilize MGMT pathways and induce cell death by targeting O^6-methylguanine adducts. Consequently, lowered MGMT levels (as an effect of epigenetic silencing) result in a greater chemotherapeutic response.

Different direct and indirect methods can address MGMT status in histological samples. MGMT enzymatic activity can be measured from frozen tumor specimens; however, this intricate processing technique and the potential contamination by non-glioma cells make this method not valuable for daily clinical practice (4). Detection of MGMT protein levels by immunohistochemistry has been linked to survival benefits in glioma patients with conflicting results; low interobserver correlation and contamination by non-neoplastic tissue are some possible explanations for such determinations. MGMT mRNA levels can be measured from fresh samples, but this implies a challenging technique with challenging to interpret results (5).

MGMT promoter hypermethylation correlates with prolonged overall (OS) and progression-free survival (PFS) in glioblastoma patients receiving alkylating agents (2, 6) and constitutes a routine molecular analysis in many institutions. Several techniques can study MGMT promoter methylation status; most of these methods are based on an initial DNA treatment by bisulfite. This reaction induces different conversions depending on whether the nucleotides are or are not methylated. Gel-based methylation-specific polymerase (MSP) is the most commonly used test, which is qualitative in nature. By contrast, pyrosequencing (PSQ) provides quantitative information on the percentage of CpGs methylation, and it has been recently validated in a meta-analysis as a prognostic tool that correlates with OS and PFS (7). Additional methylation-specific assays can be reviewed in (5).

Although DNA methylation patterns remain relatively constant, they can change in response to microenvironmental factors (8). Several groups have attempted to quantify these genetic alterations focusing on recurrent glioblastoma to detect a possible mechanism that explains universal treatment failure and tumor recurrence, albeit with inconsistent results (9, 10).

In this study, 25 matched pre and post-treatment glioblastoma samples were assessed to identify modifications of the proportion of methylated CpG dinucleotides in the CpG island. We postulate that the percentage of methylated residues may decrease after several months influenced by chemoradiotherapy. Thus, this study aims to explore the data quantitatively by employing pyrosequencing, which may reveal undetectable changes when considering methylation as a categorical variable.

Methods

Tissue samples were retrospectively obtained from patients diagnosed and treated with two or more interventions in our institution between 2009 and 2020. Only patients with confirmed histology of glioblastoma in both specimens were selected for genetic analysis, thus, excluding radionecrosis or malignant transformation from lower-grade gliomas. Clinical data included sex, age at diagnosis, IDH-status, type of treatment after the first surgery, OS, and PFS in months. OS was defined as the period from initial diagnosis until death or last follow-up, and PFS as the interval from diagnosis to objective tumor recurrence in neuroimaging based on RANO criteria (11). Finally, postprogression survival (PPS) was defined as the time elapsed from tumor recurrence to death.

Pathologic specimens were preselected to avoid contamination from necrotic and inflammatory tissue. Areas with the highest amount of neoplastic cells were marked by our pathologist and were manually dissected under microscopic guidance for DNA analysis. DNA extraction from formalin-fixed paraffin-embedded samples was performed using the Qiagen QIAmp DNA FFPE Tissue Kit (Qiagen, Valencia, CA), and the DNA amount was measured via the nanodrop lite technique. Bisulfite conversion was accomplished according to the Qiagen EpiTec Fast protocol. Afterward, PCR amplification of the MGMT promoter gene sequence was completed using the therascreen MGMT Pyro Kit. Before the final pyrosequencing analysis on the PyroMark Q24 System, samples were prepared as stated by the manufacturer´s handbook. Pyrosequencing results of the 4 CpG analyzed dinucleotides were controlled by samples with pre-established results (i.e., methylated and unmethylated) to detect possible incomplete bisulfite conversion, which can result in biased methylation quantification.

MGMT promoter methylation was classified according to recently designed cut-off levels (12), in unmethylated (< 9%), intermediate (9–29%), and highly methylated (> 29%) and also measured as a continuous variable, indicated by the average percentage value of the examined CpGs. Statistical analysis was performed using SPSS 22.0 (IBM, Armonk, NY). We initially investigated our data distribution pattern by the Shapiro-Wilk test. Based on our results, non-parametric tests were used. Wilcoxon signed-rank test was employed to compare the methylation status of matched samples from the same patient after treatment influence. The log-rank test was used to establish a correlation between survival data and methylation status. p values less than or equal to 0.05 were considered significant.

Results

In our institution, 35 patients with glioblastoma underwent surgical treatment at recurrence between 2009 and 2020. Four cases of malignant transformation from anaplastic gliomas and a case of radionecrosis were excluded from the analysis. In 5 patients, there was no tissue availability, and thus they were neither included. Therefore, MGMT promoter methylation status was examined in 25 paired samples.

The mean age of the cohort was 54 years (range 36–70). IDH1-mutation was detected in 2 cases (8%). All patients received microsurgery assisted by different surgical tools, including neuronavigation, 5-ALA

fluorescence, or intraoperative MRI depending on the period. Based on an early postoperative MRI, gross total resection was achieved in 23 out of 25 patients. Twenty-one (84%) patients completed the Stupp protocol, consisting of concomitant chemoradiotherapy followed by six cycles of adjuvant temozolomide. The remainder (16%) did not accomplish all the cycles because of early radiological progression or chemotherapy toxicity. Median PFS and OS were 13 and 25 months, respectively.

Eight (32%) primary malignancies were considered to be highly methylated; 11 (44%) were intermediate, and 6 (24%) were unmethylated. The subsequent histological analysis of paired cases revealed that methylation status remained in the same category in 15 (60%) and changed in 10 (40%). Modification in methylation status was registered in both directions, equally distributed from higher grade to lower than vice versa. Patients ´ main clinical characteristics are illustrated in Table 1.

The median value of MGMT promoter methylation status decreased after adjuvant treatment from 22.25–16.15%. Wilcoxon signed-rank test was used to compare the methylation status of matched samples without obtaining statistical significance in the difference between paired values ($p = 0.872$)

Log-rank test was employed to determine the relationship between the degree of methylation and survival data. A not statistically significant trend towards the proportion of methylation in primary samples and OS was found. Median OS was 32, 20, and 19 months in the highly methylated, intermediate, and unmethylated groups, respectively ($p = 0.102$). Lower degrees of correlation were obtained when studying primary methylation and PFS (20, 11, and 10 months, respectively; $p = 0.187$). Kaplan-Meier survival curves for OS and PFS are illustrated in Fig. 1. Tumors whose methylation status at recurrence was intermediate were significantly associated with PPS (10, 16, and 9 months, respectively; $p = 0.030$). Figure 2represents percentual changes in MGMT methylation status of the whole cohort.

Table 1
Patients ´clinical characteristics and methylation status in 25 paired samples

Pt	Gender,age	Resec	IDH	Pr. Meth	CRT	Sec. Meth	PFS	OS	Dif
1	M, 47	GTR	WT	HM (57.5%)	C	HM (59.2%)	25	36	+ 1.7%
2	M, 66	GTR	WT	HM (30.2%)	C	UM (7.0%)	16	23	-23.2%
3	M, 45	GTR	WT	IM (13.5%)	C	IM (14.5%)	8	17	+ 1.0%
4	F, 69	GTR	WT	HM (82.2%)	C	HM (92.7%)	47	56	+ 10.5%
5	M,60	GTR	WT	IM (12.7%)	C	HM (58.2%)	11	20	+ 45.5%
6	M,53	GTR	WT	HM (46.5%)	C	HM (36.7%)	28	97	-9.75%
7	F, 61	GTR	WT	IM (14.0%)	C	IM (21.5%)	12	29	+ 7.5%
8	F, 66	GTR	WT	IM (22.2%)	C	IM (18.2%)	9	25	-4.0%
9	M, 49	GTR	MUT	IM (24.7%)	C	HM (40.5%)	32	77	+ 15.8%
10	F, 55	GTR	WT	IM (27.2%)	C	UM (7.5%)	10	16	-19.7%
11	M,70	GTR	WT	HM (29.7%)	C	UM (8.0%)	20	30	-21.7%
12	F, 62	STR	WT	UM (4.0%)	I	UM (6.2%)	7	13	+ 2.2%
13	F, 59	GTR	WT	UM (6.2%)	C	UM (5.5%)	13	25	-0.7%
14	F, 56	GTR	WT	IM (20.7%)	C	IM (23.0%)	12	16	+ 2.2%
15	M, 64	GTR	WT	UM (7.0%)	I	IM (12.7%)	7	12	+ 5.7%
16	F, 61	GTR	WT	IM (15.2%)	C	IM (14.0%)	9	16	-1.2%
17	M, 48	GTR	WT	UM (4.7%)	C	IM (16.5%)	26	43	+ 11.7%
18	M, 36	GTR	WT	UM (3.5%)	C	UM (6.0%)	18	26	+ 2.5%
19	M, 48	GTR	WT	IM (24.5%)	C	UM (4.7%)	9	12	-19.8%
20	F, 47	GTR	WT	IM (11.2%)	C	IM (10.0%)	16	45	-1.2%
21	M, 37	GTR	WT	UM (3.7%)	I	UM (6.0%)	10	19	+ 2.2%
22	M, 64	STR	WT	HM (74.0%)	I	IM (28.0%)	6	32	-46.0%
23	M, 51	GTR	WT	HM (40.7%)	C	HM (31.2%)	14	18	-9.4%
24	F, 34	GTR	MUT	IM (24.2%)	C	HM (46.0%)	57	67	+ 21.7%
25	F, 58	GTR	WT	HM (39.7%)	C	HM (29.7%)	103	110	-10%

Legend: Pt: patient, Resec: resection, GTR: gross total resection, STR: subtotal resection, IDH: isocitrate dehydrogenase, WT: wildtype, MUT: IDH-1 mutated, Pr. Meth: primary methylation, HM: hypermethylated,

IM: intermediate methylation, UM: unmethylated, CRT: chemoradiotherapy, C: complete Stupp, I: Incomplete Stupp, Sec. Meth: secondary methylation, PFS: progression free survival,

Discussion

Following recent and validated cut-off values (12), a change in the category of MGMT promoter methylation status between primary and recurrent glioblastoma was noted in 10 (40 %)cases. Out of these, switching from higher to lower methylation levels was equally frequent than vice versa. These results partially agree with a recent meta-analysis (9) in which a change from methylated to unmethylated was found to be almost twice as frequent. It has to be taken into account that a high proportion of studies included in this meta-analysis use the MSP technique and dichotomize tumors into methylated or unmethylated without considering the intermediate methylation category. When analyzing the methylation level as a continuum, we observed increments or decrements nearly equally distributed in our cohort. However, the median value for the whole series dropped by approximately 6% between the first and second pathologic analysis.

Survival data of patients and proportion of tumors exhibiting higher degrees of methylation of MGMT promoter gene is higher in our series than in previous reports (12–14). This may reflect our highly selective indications for re-do procedures since we do not usually consider a reoperation for incomplete resections at first surgery or PFS intervals lower than 6–9 months. Thus, we might be excluding patients with more aggressive lesions (i.e., unmethylated). Survival benefit among patients whose tumors contain a methylated MGMT promoter is well established (2). Still, to our best knowledge, this is the first study that considers methylation status as a continuous variable and investigates its correlation with survival. Similar to previous reports (9, 15, 16), we observed that methylation was more linked to OS than PFS, although statistical significance was not achieved, possibly attributable to the small size of our cohort. The association between methylation status at recurrence and PPS was unexpected. Tumors whose methylation status was intermediate were more linked to post-progression survival than hypermethylated ones. This may be again influenced by other factors such as the limited size of our study group.

Methylation status results may be dependent on the laboratory test used for its determination. MSP has been to date, the most used technique to validate MGMT methylation status changes detection (15, 17–21) however, Park et al. (22) concluded that methylation changes might remain undetectable when analyzing tumors by MSP while other techniques, such as methylation-specific multiplex ligation probe amplification (MS-MLPA) can readily uncover the downward shift of methylation in recurrent tumors. In our study, pyrosequencing was used to detect these changes, but similar results to those previously published were obtained.

On the other hand, tumor samples for methylation analysis can provide different results if not previously selected. To avoid this kind of contamination in our study, areas with a higher amount of neoplastic cells were marked and manually dissected to exclude non-neoplastic elements as previously described (17). Intratumor heterogeneity concerning epigenetic silencing of the MGMT gene has been reported by some

groups (17, 23) others have found that MGMT promoter methylation status is relatively homogenous within the tumor (18, 24) and therefore we have only examined one preselected area from each tumor. By contrast, MGMT protein expression evaluated by immunochemistry appears to have a decreasing gradient from the inner to the outer portion of the tumor (23). These data support that there is low concordance between MGMT promoter methylation and protein levels and that there may be other mechanisms involved in its expression apart from epigenetic silencing (25).

Another issue is the definition and validation of the technically and clinically relevant cut-off value for MGMT promoter methylation and the fact that most samples will be classified similarly by most tests, although different CpGs are interrogated (26).

Due to its mechanism of action, clonal selection by TMZ has been elucidated as a primary factor that could explain treatment resistance and methylation pattern reshaping within the clinical course. TMZ may deplete cell lines with low MGMT expression (i.e., hypermethylated promoter), inducing chemoresistance by selecting clones with low levels of methylation which are more resistant to alkylating drugs (16). Although exposed in a mathematical model (27), this feature remains controversial since changes from unmethylated to highly methylated status in paired samples after chemotherapy can be detected in several studies, including ours (15–17, 22, 28, 29). On the contrary, other authors found differences in the methylation pattern of recurrent glioblastoma depending on whether the patient was treated with RT followed by TMZ versus concurrent RT/TMZ (16, 20). Furthermore, it has been reported that switching from methylated to unmethylated status can happen without chemotherapy in lower-grade glioma cases (19). We consider our cohort to be very homogenous in terms of post-surgical treatment since all patients received simultaneous chemoradiotherapy, and 84% were treated with at least six cycles of adjuvant TMZ. These data suggest that epigenetic dynamics of MGMT promoter methylation status over the clinical course might be very complex and influenced by several processes that are still poorly understood. Additional mechanisms have been theorized in which chemotherapy with alkylating agents may promote de-differentiation from non-cancer stem cells to glioma stem cells, responsible for tumor growth and chemoresistance acquisition (30).

Several drawbacks need to be considered in the present study. Its small sample size limits the statistical power of our results. A future multicentre, more extensive study would be of interest, considering multi-area biopsies and other methylation measuring techniques that may disclose different conclusions regarding modifications in epigenetic changes.

Conclusions

Although MGMT promoter methylation status was examined quantitatively, we did observe both increments and decrements in paired samples after temozolomide treatment. These data suggest that clonal selection caused by alkylating agents does not entirely explain the dynamics of MGMT epigenetic changes. Factors related to MGMT tumor heterogeneity, laboratory tests used, and samples quality may

also influence our results. Despite our small cohort size, MGMT promoter methylation status seems to correlate with OS and a greater chemotherapeutic response, which impacts longer PFS.

List Of Abbreviations

MGMT
O^6-methylguanine-DNA-methyltransferase.
TMZ
temozolomide.
OS
overall survival.
PFS
progression free survival.
PPS
postprogression survival.
MSP
methylation-specific polymerase.
PSQ
pyrosequencing.
RANO
response assessment in neurooncology.
FFPE
formalin-fixed paraffin-embedded.
IDH-1
isocitrate dehydrogenase 1.
MRI
magnetic resonance imaging.
SPSS
Statistical Package for the Social Sciences.
5-ALA
5-aminolevulinic acid.
MS-MLPA
methylation-specific multiplex ligation probe amplification.
RT
radiotherapy.

Declarations

Ethics approval and consent to participate: The study protocol was approved by the institutional ethic committee (*Comité de Ética de la Investigación de las Islas Baleares; CEI-IB*). We ensure that all methods

were performed in accordance with the relevant guidelines and regulations. As our research is retrospective in nature, the institutional ethic committee *(CEI-IB*) approved the waiver for informed consent.

Consent for publication: Not applicable.

Availability of data and materials: The dataset analysed during the current study is available from the corresponding author and summarized in Table 1. Moreover, the data is available at BioProject, a INSDC member repository. https://trace.ddbj.nig.ac.jp/D-way/contents/bp/bp_submission_detail?submission_id=PSUB017363.

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Competing interests: The authors declare that they have no competing interests.

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Authors' contributions:

Conceptualization and design: VGJ, MBD, JID.

Methodology: VGJ, MBD, CGB.

Data acquisition: VGJ, MBD, CGB, OSA, VG.

Analysis and interpretation of data: VGJ, CGB, AGM.

Writing – original draft preparation: VGJ.

Writing – review and editing: MBD, JID.

All authors have approved the submitted version.

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Figures

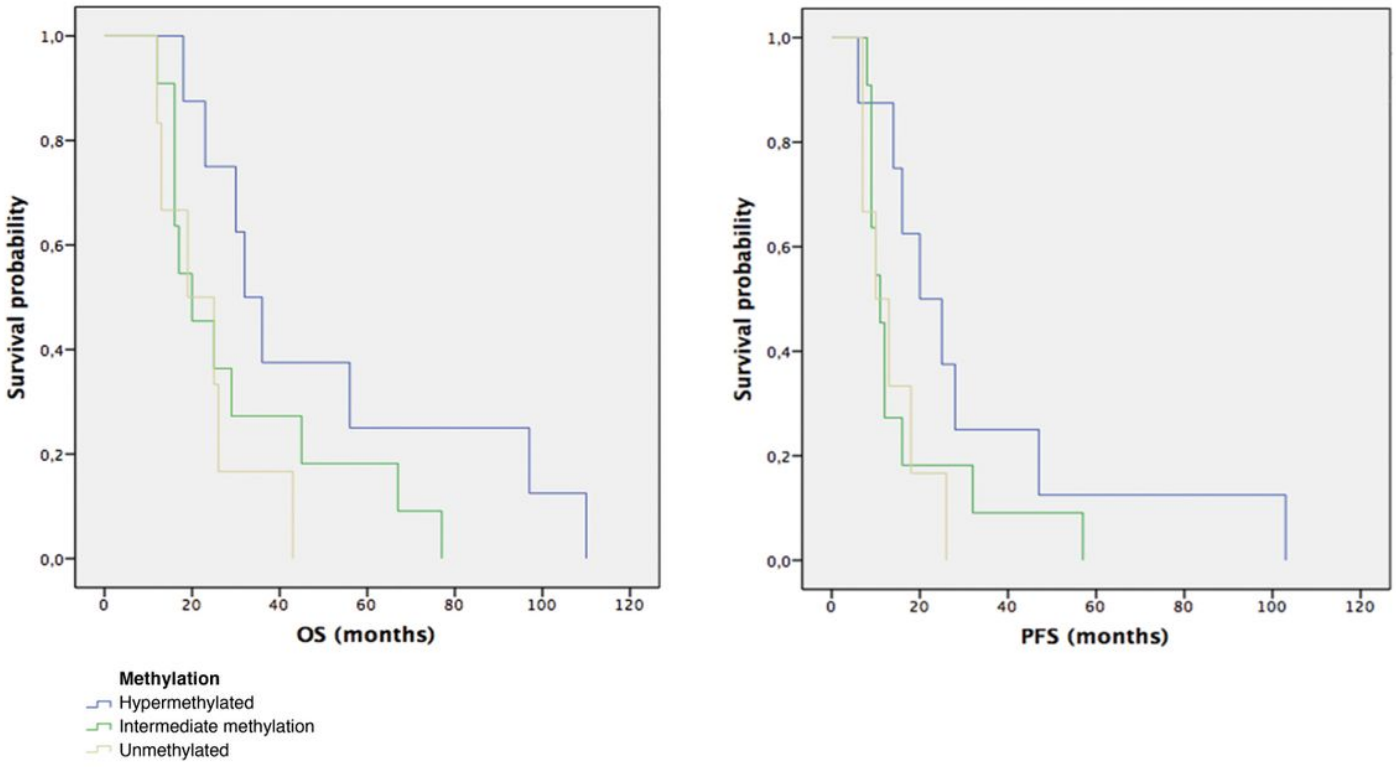

Figure 1

Kaplan-Meier OS (left) and PFS (right) functions regarding MGMT methylation status at first determination.

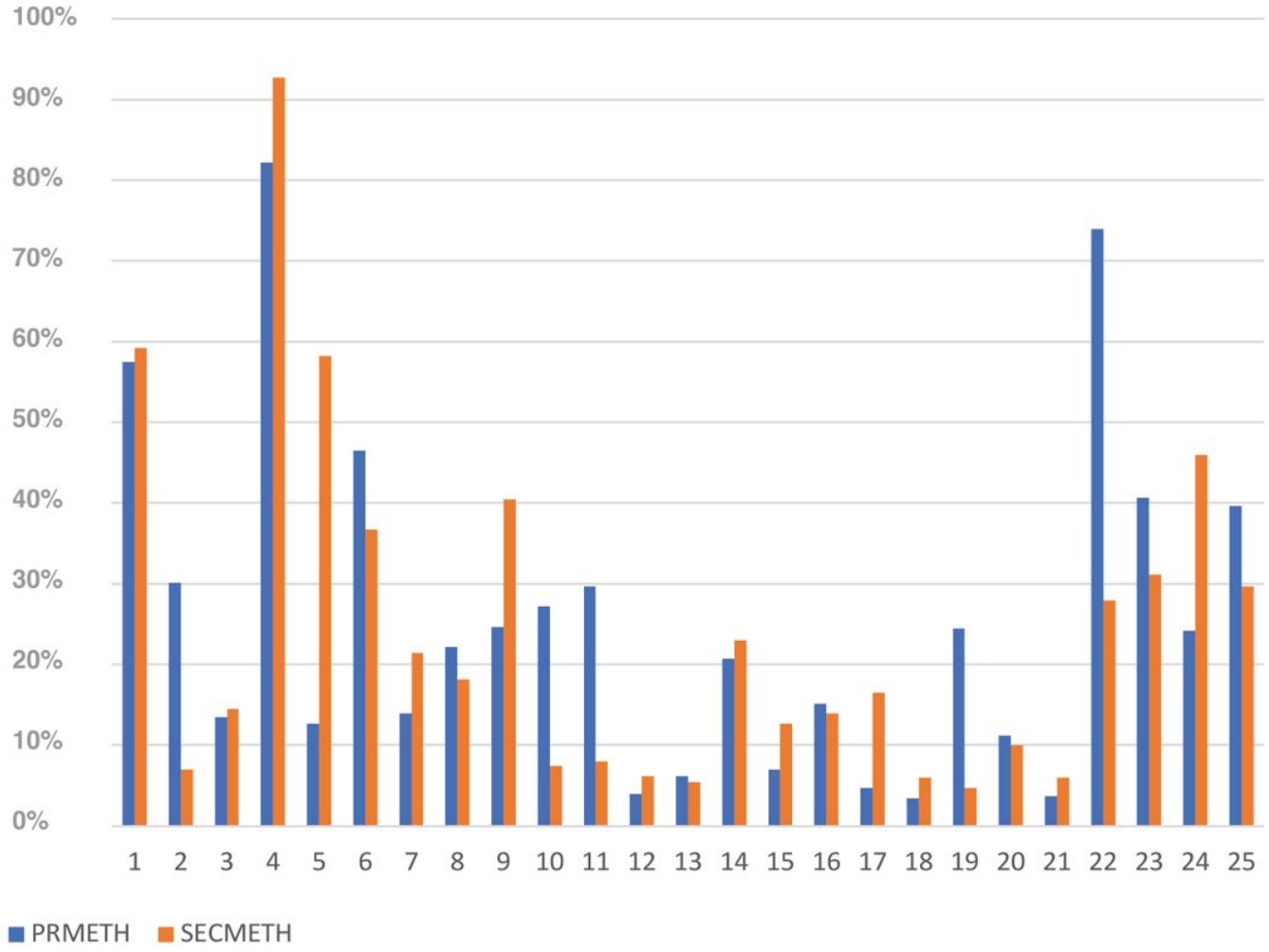

Figure 2

Quantitative analysis of MGMT promoter methylation status at first determination (blue bar) and recurrence (orange bar).