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LABORATORY INVESTIGATION

Low *MGMT* digital expression is associated with a better outcome of *IDH1* wildtype glioblastomas treated with temozolomide

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Abstract

Introduction Glioblastoma (GBM) is the deadliest primary brain tumor. The standard treatment consists of surgery, radiotherapy, and temozolomide (TMZ). TMZ response is heterogeneous, and *MGMT* promoter (*MGMTp*) methylation has been the major predictive biomarker. We aimed to describe the clinical and molecular data of GBMs treated with TMZ, compare *MGMT* methylation with *MGMT* expression, and further associate with patient's outcome.

Methods We evaluate 112 FFPE adult GBM cases. IDH1 and ATRX expression was analyzed by immunohistochemistry, hotspot *TERT* promoter (*TERTp*) mutations were evaluated by Sanger or pyrosequencing, and *MGMTp* methylation was assessed by pyrosequencing and *MGMT* mRNA expression using the nCounter® Vantage 3D™ DNA damage and repair panel.

Results Of the 112 GBMs, 96 were $IDH1^{WT}$, and 16 were $IDH1^{MUT}$. Positive ATRX expression was found in 91.6% (88/96) of IDH^{WT} and 43.7% (7/16) of IDH^{MUT}. *TERTp* mutations were detected in 70.4% (50/71) of IDH^{WT}. *MGMTp* methylation was found in 55.5% (35/63) of IDH^{WT} and 84.6% (11/13) of IDH^{MUT}, and as expected, *MGMTp* methylation was significantly associated with a better response to TMZ. *MGMT* expression was inversely correlated with *MGMTp* methylation levels (−0.506, $p < 0.0001$), and *MGMT* low expression were significantly associated with better patient survival. It was also observed that integrating *MGMT*p methylation and expression, significantly improved the prognostication value.

Conclusions *MGMT* mRNA levels evaluated by digital expression were associated with the outcome of TMZ-treated GBM patients. The combination of *MGMT* methylation and mRNA expression may provide a more accurate prediction of TMZ response in GBM patients.

Keywords Glioblastoma · *MGMT* · IDH · Temozolomide · NanoString · Brazil · Biomarkers · Methylation

Introduction

According to the World Health Organization (WHO), GBM, grade IV glioma, is the most common and deadliest primary malignant brain tumor [1]. It accounts for 48.3% of gliomas in adults and 14.6% of all Central Nervous System (CNS)

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tumors [1]. GBM's incidence rate for 2012–2016 was estimated at 3.22 per 100.000 population, and it is more common in males (ratio: 1.58) [1].

The molecular analysis of GBM and histopathological features have substantially improved tumor classification and prediction of outcome, allowing better treatment strategies and decisions for GBM patients. Currently, the WHO 2016 classification for CNS tumors comprises molecular markers for the classification of diffuse gliomas, such as isocitrate dehydrogenase *(IDH)* mutational status [2].

Primary GBM arise "de novo" and lacks mutations in the *IDH1/2* genes (IDH^{WT}). GBM IDH^{WT} accounts for 90% of cases, usually occurs in older patients, and is associated with a dismal prognosis, with five years median OS of 15 months and only 6.8% of them survive more than 5 years after diagnosis [1]. Approximately 10% of GBM cases are classified as secondary GBMs, which arises from a malignant progression from lower-grade gliomas (grades II and III) and harbor mutations in *IDH1/2* genes (IDH^{MUT}). GBMs IDH^{MUT} are associated with a better outcome with 5-years OS about 31 months for patients submitted to standard therapy [2].

TERT (Telomerase Reverse Transcriptase) and *ATRX* (alpha-thalassemia/mental retardation syndrome X-linked) mutational status are also crucial for the classification and prognosis of gliomas [2]. Each subgroup can develop different specific mechanisms that result in increased telomeres length. GBMs harboring *TERT* promoter and *IDH* mutations exhibit alternate telomere elongation (ALT) due to loss-of-function mutations in *ATRX* [3–5]. GBM IDH^{MUT} carries *ATRX* mutations in approximately 71% of the cases, and GBM IDH^{WT} carries *TERT* promoter mutations in approximately 72% of the cases [2].

GBM's standard therapy is surgery followed by radiation, with concomitant and adjuvant temozolomide (TMZ), an oral alkylating agent [6]. However, the prognosis is very reserved, and it is estimated that at least 50% of TMZ-treated patients do not respond to chemotherapy [7]. Some patients are short term survival (STS) ($OS \leq 12$ months) and others long term survival (LTS) ($OS \geq 36$ months) [8], and the overall survival (OS) is influenced by treatment schemes and patients features, such as *IDH1/2* and *TERT* mutations, *MGMT* methylation [2, 6].

The O-6-methylguanine DNA methyltransferase (*MGMT*) gene codes a DNA repair enzyme that removes DNA damages promoted by alkylating agents such as TMZ. Currently, *MGMT* status is the major predictive biomarker for TMZ response in GBMs [9]. The transcriptional control of *MGMT* gene expression is mainly regulated at the epigenetic level by promoter methylation [10]. The presence of methylation in the *MGMT* promoter impairs the production of the MGMT DNA repair enzyme, and the TMZ exposure results in DNA damage-inducing tumor cell death [11]. Patients with methylated *MGMT* promoter show a better response to TMZ, while those with unmethylated *MGMT* promoter do not benefit from the same treatment (median OS: 23.4 vs. 12.6 respectively) [11–13].

The methylated *MGMT* promoter occurs in 30–50% of GBMs IDH^{WT} [14]. Methylation analysis has been currently conducted by many different methodologies, all of them involving amplification steps, including quantitative methylation-specific PCR (qMSP), methylation-sensitive high resolution melting (MS-HRM), next generation sequencing (NGS), and the most widely used is pyrosequencing (PSQ) [9]. Despite extensive attempts to standardization, there is a wide variation in sensitivity, specificity, and it was also observed significant discordance between different methods [15]. NanoString technology is direct digital detection system, which enables both highly sensitive and reproducible multiplexed gene quantification without amplification, PCR-free. It measures nucleic acid using fluorescent molecular barcode probes which bind directly to chosen targets: mRNA, miRNA, or DNA. Since each probe as 50 bp long its suitable for analyzing formalin-fixed paraffin-embedded (FFPE) samples [16, 17].

In this study, we aimed to report the clinical and molecular characteristics of TMZ-treated GBM patients from a Brazilian institution and to compare the *MGMT* methylation with *MGMT* gene expression employing and highly sensitive PCR-free quantification method and to associate the molecular data with patients' outcome.

Material and methods

Patients

In the present study, epidemiologic, pathological, and clinical data of 112 adult GBM patients treated according to Stupp protocol (concomitant radiotherapy plus TMZ followed by adjuvant TMZ) [6], at Barretos Cancer Hospital (BCH) from 2008 to 2018 were retrospectively collected. The histology of all cases was reviewed and confirmed by experienced neuropathologists (MMM). The overall survival (OS) was estimated from histological diagnosis to date of death or last follow-up. The information on age at diagnosis, gender, tumor location, Eastern Cooperative Oncology Group (ECOG), Karnofsky Performance Status (KPS), subtype, and radio/chemotherapy is summarized in Table 1. The present study was approved by the local ethics committee (Barretos Cancer Hospital IRB/Project No. 1604/2018), which bestowed the exemption of informed consent due to the retrospective nature of the study. All methods were performed in accordance with the relevant guidelines and regulations (Declaration of Helsinki).

All subsequent immunohistochemistry and molecular analysis were performed in treatment-naive GBM samples.

Table 1 Clinical and pathological features of glioblastomas patients according to *IDH* mutational status

Variables	Parameters	GBM IDH^{WT} (N = 96)		GBM IDH^{MUT} (N = 16)		p-value
		N	%	N	%	
Age at diagnosis	Median (range)	54 (29–75)		42 (25–60)		**0.001**
Gender	Female	41 (42.7%)		8 (50.0%)		0.586
	Male	55 (57.3%)		8 (50.0%)		
Tumor location	Frontal	19 (19.8%)		9 (56.3%)		**0.005**
	Temporal	35 (36.4%)		3 (17.6%)		
	Parietal	23 (24.0%)		0 (0%)		
	Multiple	14 (14.6%)		3 (17.6%)		
	Other	5 (5.2%)		1 (5.8%)		
ECOG	0	40 (41.7%)		7 (43.8%)		0.828
	1	16 (16.7%)		4 (23.5%)		
	2	4 (4.2%)		0 (0%)		
	Missing	36		5		
KPS	< 70	3 (5.0%)		0 (0%)		0.863
	≥ 70	57 (95.0%)		11 (100%)		
	Missing	36		5		
Surgical resection	Biopsy	2 (2.1%)		1 (5.9%)		0.635
	Partial	59 (61.5%)		10 (58.8%)		
	Complete	30 (31.3%)		5 (35.3%)		
	Missing	5		0		
Histological subtype	Classic GBM	93 (96.8%)		13 (81.2%)		**0.037**
	Giant cell GBM	3 (3.2%)		2 (12.5%)		
	Small cell GBM	0 (0%)		1 (6.3%)		
2nd resection	No	73 (76.0%)		13 (82.4%)		0.760
	Yes	23 (24.0%)		3 (17.6%)		
Radio/Chemotherapy	No	34 (35.4%)		5 (35.3)		0.746
	Yes	62 (64.6%)		11 (64.7%)		
≥ 6 cycles TMZ	No	44 (45.8%)		3 (23.5%)		**0.042**
	Yes	52 (54.2%)		13 (76.5%)		
2nd Radiotherapy	No	94 (97.9%)		12 (75.0%)		**0.004**
	Yes	2 (2.1%)		4 (25.0%)		
Status	Alive (active disease)	8 (8.3%)		6 (41.2%)		**0.008**
	Death (cancer-specific)	78 (81.3%)		10 (58.8%)		
	Death (other causes)	10 (10.4%)		0 (0%)		

ECOG Eastern Cooperative Oncology Group, *KPS* Karnofsky Performance Status, *GBM* Glioblastoma
Bold, significant values

IDH1 R132H and ATRX immunohistochemistry

Immunohistochemistry was performed on FFPE 4 µm sections. IDH1 and ATRX reactions were performed on a BenchMark Ultra (Ventana Medical Systems) using the OptiView DAB IHQ Detection Kit (ROCHE) and UltraView DAB IHQ Detection kit (ROCHE), respectively. Slides were incubated with the primary antibodies mouse anti-human IDH1 R132H (DIA-H09–Dianova, dilution 1:50) and rabbit anti-human ATRX (Sigma HPA001906, dilution 1:300) for 32 min. IDH1 and ATRX immunostaining were scored as previously reported [18, 19].

DNA and RNA isolation

DNA and RNA from FFPE tissues were retrieved from 10 µm slides, after careful macrodissection of tumor area, ensuring more than 80% of neoplastic cells and absence of necrosis and microvascular proliferation. DNA was isolated using the QIAamp DNA Mini Kit (Qiagen, Venlo, The Netherlands) following the manufacturer's instructions, quantified by NanoDropVR 2000 (Thermo Scientific, Waltham), and stored at – 20 °C for further applications.

RNA was isolated using the deparaffinization solution (Qiagen, Venlo, The Netherlands) and the RNeasy Mini Kit

(Qiagen, Venlo, The Netherlands) following the manufacturer's instructions. The total RNA was quantified by Qubit 2.0 Fluorometer using the Qubit RNA HS Assay kit (Life Technologies, Thermo Fisher Scientific, EUA) and stored at – 80 °C for further applications.

Detection of *TERT* promoter mutations

The hotspot mutations c.-146: G > A and c.-124: G > A (C250T and C228T, respectively) regions of the *TERT* promoter region were evaluated by PCR followed by direct Sanger sequencing, as previously reported by our group [20]. Furthermore, *TERT* mutational analysis was also carried out by pyrosequencing as previously reported [21] in a subset of samples, with inconclusive results by Sanger sequencing. Results were analyzed using the PyroMark Q96 software and samples were considered mutated when the percentage of mutated alleles was above 5%.

Bisulfite pyrosequencing for *MGMT* methylation analysis

The methylation status of four CpG sites in the *MGMT* promoter was determined by pyrosequencing, as previously reported [22, 23]. Briefly, tumor DNA (200–400 ng/µL) was subjected to bisulfite conversion using the EpiTect Bisulfite Kit (Qiagen, Venlo, The Netherlands), bisulfite-converted DNA (50–100 ng) was amplified using MGMT Pyro Kit (Qiagen, Venlo, The Netherlands) and PCR products analyzed on a PyroMark Q24 (Qiagen, Venlo, The Netherlands) using reagents and primers from the commercial kit (MGMT Pyro kit; Qiagen, Venlo, The Netherlands), and following the manufacturer's recommendations. All pyrosequencing runs included a 100% methylated commercial DNA (Qiagen, Venlo, The Netherlands) and an unmethylated DNA (genomic DNA isolated from healthy subjects' peripheral blood). The percentage of methylated alleles was calculated as the mean value of the methylation percentage obtained at each CpG investigated, and samples were classified as unmethylated when below threshold (10%), according to previous studies [22, 23].

MGMT expression by NanoString

MGMT mRNA expression was evaluated by the nCounter® Vantage 3D™ DNA Damage and Repair Panel (NanoString Technologies, Seattle, WA, USA), which contains 180 genes related to major damage and repair pathways, including *MGMT*. Total RNA (100 ng) was hybridized with probe pools, hybridization buffer, TagSet and incubated at 67 °C for 23 h 15 min. Hybridization, immobilization and purification were automated conducted in the PrepStation (NanoString Technologies, Seattle, WA, USA). Fluorescent barcodes were scanned by nCounter® Digital Analyzer (NanoString Technologies, Seattle, WA, USA), considering 555 fields of view (FOVs) to capture all gene counts. Raw data were collected and pre-processed by nSolver™ Analysis Software v4.0 (NanoString Technologies, Seattle, WA, USA). Standardized quality control (QC), including imaging QC, binding density, limit of detection QC, positive and negative controls QC, was conducted for all samples. Only samples fulfilling all QC were eligible for data analysis. Data analysis was conducted in the R statistical environment (v3.6.2). Batch effects were checked by Quantro (v1.18.0) [24]. Data normalization was performed using the NanoStringNorm package (v1.2.1) [25] applying quantile normalization and log2 transformation, followed by differential expression analysis. Samples were binarily classified in low and high expression according to *MGMT* expression's median value.

Statistical analysis

The samples were characterized using frequency tables for qualitative variables and measured central tendency (mean, median) and dispersion (standard deviation, minimum, and maximum) for quantitative variables.

Chi-square or Fisher's Exact and Mann–Whitney tests were used to verify the univariate association between clinical characteristics and molecular markers. For survival analysis, Kaplan Meier curves (Log Rank test) and Cox regression analysis were performed using the IBM SPSS Statistics for Windows version 21.0 (IBM, Armonk, NY, USA). GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA) was employed to construct graphics. Significance was considered as two-tailed p value < 0.05.

Results

Clinical, pathological, and molecular features of GBM

A total of 112 patients diagnosed with GBM and treated with radiotherapy and TMZ were characterized. IHC analysis showed that 85.7% (96/112) of samples were $IDH1^{R132H}$ negative (IDH^{WT}) (Supplementary Fig. 1A), and 14.3% (16/112) were $IDH1^{R132H}$ positive (IDH^{MUT}) (Supplementary Fig. 1B).

Cases *IDH1* positive were significantly associated with younger age at diagnosis (median age: 42 years old, ranging from 25 to 60 years) when compared with IDH^{WT} (median age: 54 years, ranging from 29 to 75 years) (p = 0.001) (Table 1). Moreover, temporal tumor localization was found in 35 (36.4%) of IDH^{WT}, and frontal localization was found in 9 (56.3%) of IDH^{MUT} (p = 0.005), histology classic

GBM was found in 93 (96.8%) of *IDH*^{WT} and 13 (81.2%) of *IDH*^{MUT} (p = 0.037), and most patients in both groups did not have additional radiotherapy treatment following Stupp protocol 94 (97.9%) in *IDH*^{WT} and 12 (75.0%) in *IDH*^{MUT} (p = 0.004) (Table 1). No significant association was found in other features (Table 1).

ATRX expression was found in 91.6% (88/96) of *IDH*^{WT} and 43.7% cases (7/16) of *IDH*^{MUT} (Supplementary Fig. 1C and D; Table 2). The *TERT* promoter mutation (*TERTp*) analysis was conclusive for 79 GBMs, due to the low quantity or quality of DNA of the remaining 33 cases. We detected the presence of *TERTp* mutations (c.-124:G > A or c.-146:G > A) in 70.4% (50/71) of *IDH*^{WT} while all *IDH*-^{MUT} were wildtype for *TERTp* (Table 2) and (Supplementary Fig. 2). The most common *TERTp* mutation was the c.-124: G > A, detected in 64.0% (32/50), and the c.-146: G > A was found in 36.0% (18/50) of GBMs.

MGMT status: promoter methylation and mRNA expression levels

We further evaluated the *MGMT* promoter (*MGMTp)* methylation by pyrosequencing, and conclusive results were obtained for 76 GBMs (Fig. 1a and Supplementary Fig. 3). *MGMTp* was considered methylated in 55.5% (35/63) of *IDH*^{WT} and 84.6% (11/13) of *IDH*^{MUT} (Fig. 1b and Table 2).

The *MGMT* mRNA expression levels by NanoString were conclusive in 95.5% (107/112) of cases. The *MGMT* mRNA median expression [− log2] = 5.25; range 2.81–6.91) (Fig. 1a). High *MGMT* expression was observed in 52.2% of GBM *IDH*^{WT} cases (48/92), while most of *IDH*^{MUT} cases showed low *MGMT* expression lower (66.7%, 10/15) (p = 0.176; Table 2).

Low levels of mRNA were found in patients with *MGMT* promoter methylated, and high levels of *MGMT* mRNA were observed in unmethylated cases (Fig. 1a, c). Inverse correlation between *MGMT* methylation and expression analysis (− 0.506) (p < 0.0001) was found (Fig. 1d).

Association of molecular features and patients' survival

Overall, GBM *IDH*^{MUT} presented significantly higher overall survival (OS) compared with GBM *IDH*^{WT} (35.4 months vs. 15.5 months; p < 0.001, Supplementary Fig. 4A), respectively. Furthermore, *IDH*^{WT} TMZ-treated cases with ≥ 6 cycles presented significantly higher median OS compared with GBM cases *IDH*^{WT} TMZ-treated with < 6 cycles (17.7 months vs. 10.1 months; p < 0.0001, Supplementary Fig. 4b).

We further evaluated the IDH^{WT} or primary subset of GBMs separately. We observed that *MGMT* methylated patients had significantly higher median OS (17.5 months vs. 12.0 months; p = 0.0001, Fig. 2a) compared to patients with unmethylated *MGMT*. It was also observed that patients with lower median mRNA expression had higher median OS than patients with *MGMT* mRNA higher median expression (17.9 months vs. 13.5 months; p = 0.0003, Fig. 2b).

In a univariate analysis, we observed that patients treated with ≥ 6 cycles of TMZ and add partial surgical resection were significantly associated with OS (HR = 0.32, p < 0.001 CI95% 0.21–0.51) and (HR = 2.00, p = 0.004 CI95% 1.24–3.21), retrospectively (Supplementary Table 1 and Fig. 3). We observed that methylated promoter and mRNA expression of *MGMT* were also significantly associated with better patient outcome (HR = 0.32, p = 0.0002 CI95% 0.17–0.58) and (HR = 0.44, p = 0.0004

Table 2 Molecular features of glioblastomas patients according to *IDH* mutational status

Molecular alterations	Parameters	GBM *IDH*^{WT} (N = 96)		GBM *IDH*^{MUT} (N = 16)		p-value
		N	%	N	%	
ATRX	Positive	88	91.6	7	43.7	
	Negative	6	6.2	9	56.3	**< 0.0001**
	Missing	2		0		
TERTp	Wild-type	21	29.6	8	100.0	
	Mutated	50	70.4	0	0.0	**0.001**
	Missing	25		8		
MGMTp methylation	Unmethylated	28	44.5	2	15.4	
	Methylated	35	55.5	11	84.6	**0.050**
	Missing	33		3		
MGMT expression	Low	44	47.8	10	66.7	
	High	48	52.2	5	33.3	0.176
	Missing	4		1		

Bold, significant values

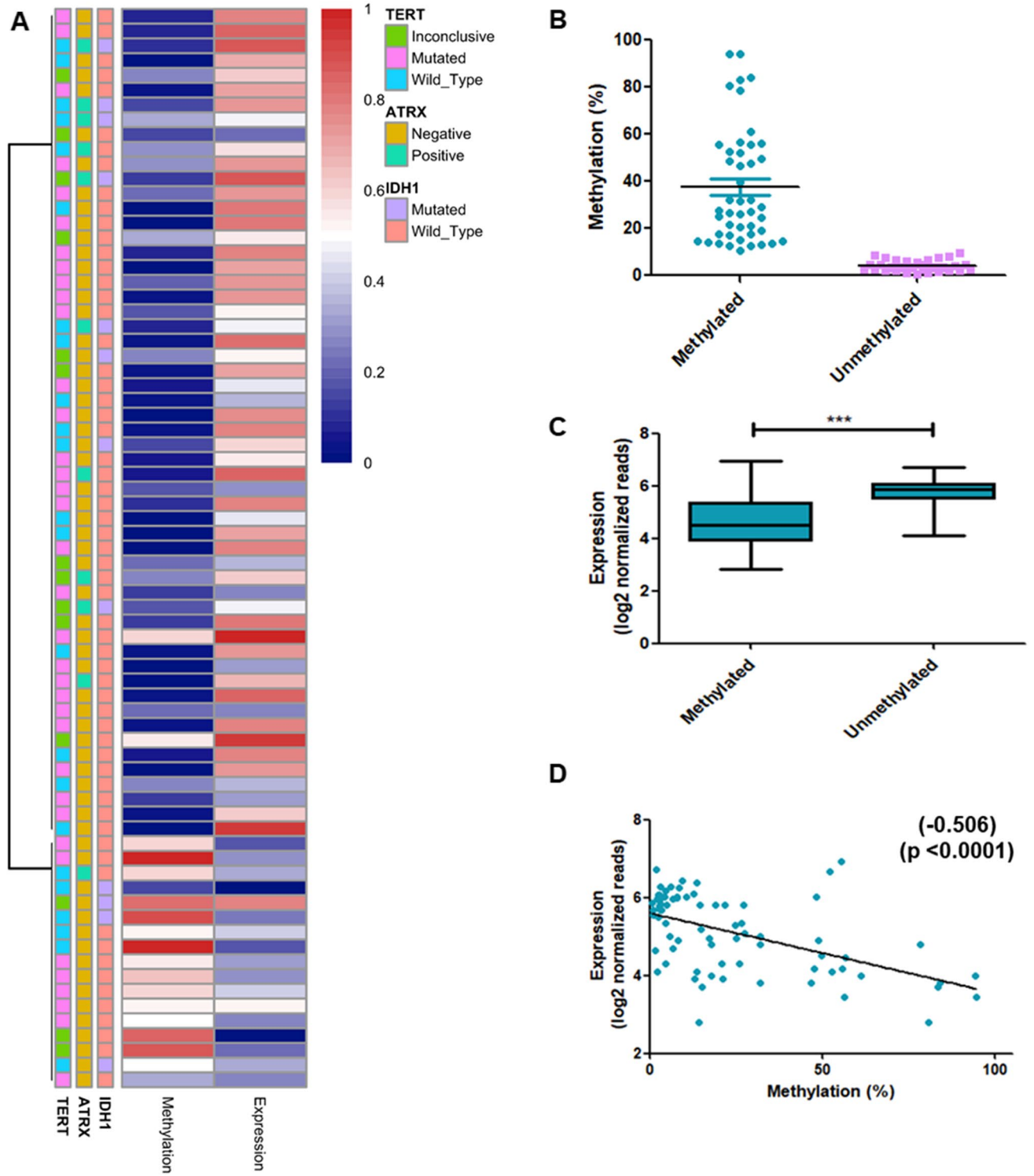
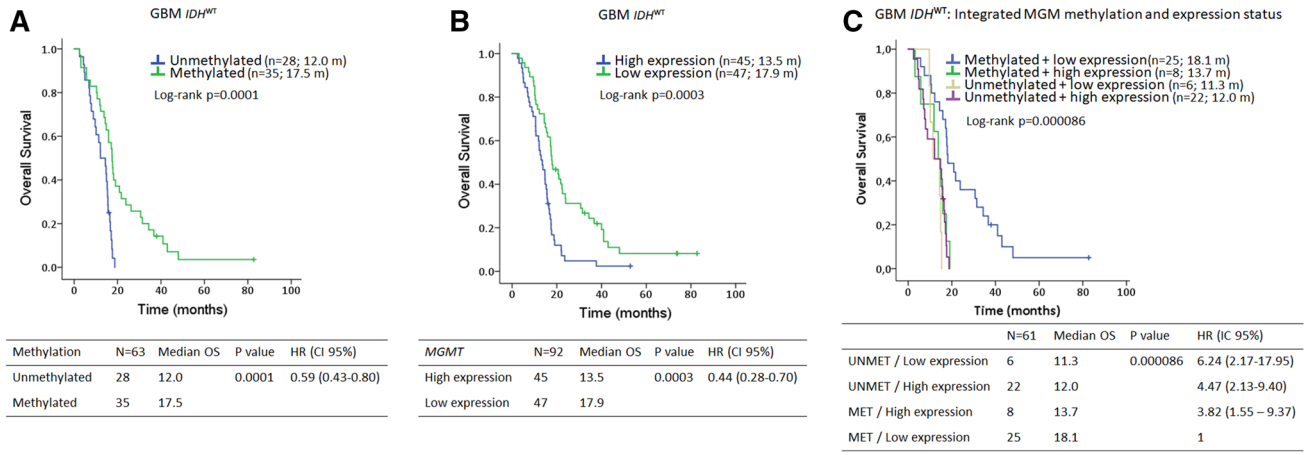


Fig. 1 **a** Molecular (*IDH1*, *ATRX*, and *TERT*) and *MGMT* status of methylation and mRNA expression. **b** Distribution of methylation levels in *MGMTp* methylated (≥10% methylation) and unmethylated cases (<10% methylation). **c** Boxplots of correlation (T-test). ***Significant differences ($p < 0.0001$) and in **d** Correlation between *MGMTp* methylation and expression (Spearman test)



Methylation	N=63	Median OS	P value	HR (CI 95%)
Unmethylated	28	12.0	0.0001	0.59 (0.43-0.80)
Methylated	35	17.5		

MGMT	N=92	Median OS	P value	HR (CI 95%)
High expression	45	13.5	0.0003	0.44 (0.28-0.70)
Low expression	47	17.9		

	N=61	Median OS	P value	HR (IC 95%)
UNMET / Low expression	6	11.3	0.000086	6.24 (2.17-17.95)
UNMET / High expression	22	12.0		4.47 (2.13-9.40)
MET / High expression	8	13.7		3.82 (1.55 – 9.37)
MET / Low expression	25	18.1		1

Fig. 2 Kaplan–Meier curves for the overall survival of GBM patients. **a** Overall survival curves according *MGMT* methylation status in GBM *IDH*^{WT} (Median OS Unmethylated = 12.0 months; Median OS Methylated = 17.5 months). **b** Overall survival curves according *MGMT* mRNA expression in GBM *IDH*^{WT} (Median OS High expression = 13.5 months; Median OS Low expression = 17.9 months). **c** Kaplan Meir curves based on both *MGMT* methylation and mRNA expression status. The tables provide the number of cases (N), median overall Survival (OS), "p" value (Log Rank test) and the Hazard Ratio (HR) 95% of confidence interval. Survival time is presented in months; p values are related to Log-rank test results

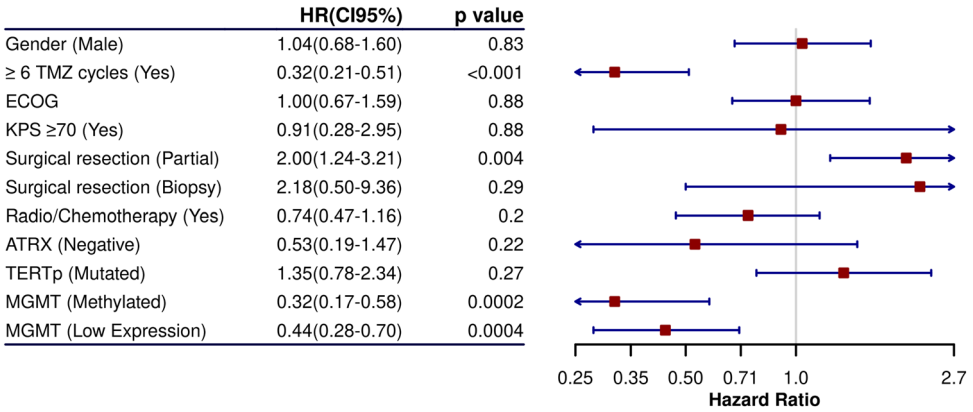


Fig. 3 Forest plot of the clinical and molecular features considered in the univariate analysis. The red squares and the blue horizontal lines indicate hazard ratios and 95% confidence intervals, respectively. *TMZ* temozolomide; *ECOG* Eastern Cooperative Oncology Group; *KPS* Karnofsky Performance Status; *TERTp TERT* promoter. Vertical line represents hazard ratio equal to 1. The score is calculated by summing all the points for a given patient, with a higher score indicating a greater risk of death

CI95% 0.28–0.70), respectively (Supplementary Table 1 and Fig. 3). The other clinical and molecular features (ATRX and *TERTp* mutations) were not significantly associated with GBM *IDH*^{WT} patient's OS (Supplementary Table 1 and Fig. 3).

Due to the different number of cases with *MGMT* methylation and expression data available, two multivariate analysis were done (Supplementary Table 1). In both analyses' patients that underwent ≥ 6 cycles of TMZ exhibited better outcome (HR = 0.34, p < 0.0001; CI95% 0.21–0.55) and (HR = 0.36, p = 0.001; CI95% 0.20–0.65), retrospectively (Supplementary Table 1). We also observed that lower *MGMT* mRNA expression and methylated *MGMT* showed a better outcome for patients (HR = 0.56, p = 0.017; CI95% 0.35–0.90) and (HR = 0.39, p = 0.005; CI95% 0.21–0.75), respectively (Supplementary Table 1).

Next, we evaluated the 61 *IDH*^{WT} cases with available information of both *MGMT* methylation and *MGMT* mRNA expression status. The integrated analyses showed that *IDH*^{WT} patients with concomitant *MGMT* methylated status and lower expression had higher overall survival (18.1 months) and lower hazard ratio compared to other subgroups: methylated and higher expression (13.7 months; HR = 3.82, p = 0.003518; CI95% 1.55–9.37); unmethylated and lower expression (11.3 months; HR = 6.24, p = 0.000689; CI95% 2.17–17.95); unmethylated and higher expression (12.0 months; HR = 4.47, p = 0.000076; CI95% 2.13–9.40) (Fig. 2c).

Discussion

In the present study, we describe the clinical and molecular (*IDH1, ATRX*, and *TERT*) characteristics of 112 Brazilian GBMs treated with radiotherapy and temozolomide-based chemotherapy (TMZ). We compared *MGMT* methylation and mRNA expression by Nanostring and showed that both assays were significantly associated with GBM IDH^{WT} outcome, with better survival discrimination when combining both *MGMT* assays.

In our cohort, IDH^{WT} represented 85% of cases, with a median age of 54 years and median OS of 15.5 months (ranging from 2.4 to 82.7 months), whereas GBM IDH^{MUT}, or astrocytoma, IDH mutant, WHO grade IV, as recently renamed in the cIMPACT-NOW updates that will constitute upcoming WHO 2021 [26], represented 15% cases, with a median of age of 42 years, and showed a 35.4 months OS (ranging from 9.5 to 76.41 months). This series is lightly younger than the 2016 WHO Classification of the Central Nervous System Tumors, with a median age at 62 years and a median OS of 15 months [2]. On the other hand, GBMs IDH^{MUT} (astrocytoma, IDH mutant, WHO grade IV) occurred in younger patients (median 44 years) and exhibited a median OS of 31 months [2].

Concerning the other molecular features, a loss of nuclear ATRX expression was observed in 6.2% of IDH^{WT} and 56.3% of IDH^{MUT}. These results agree with other studies that reported ATRX loss between 7–11% of GBM IDH^{WT} and 53–69% of GBM IDH^{MUT} [7, 27]. *TERTp* mutation were observed in 70.4% of IDH^{WT} and 0% of IDH^{MUT} cases, in accordance with international literature [2–4, 20, 28]. At variance with these studies, we did not find an association of ATRX and *TERTp* status with the patient's outcome.

This study's major aim was to compare two methodologies of *MGMT* status analysis and their role in GBM patients' response to temozolomide. Much evidence supports that the *MGMT* status in GBM is associated with patients prognostic and response to TMZ; however, the variation in detection methods and cutoff definitions remains the major challenge for consensus between laboratories [9, 29]. *MGMT* methylation analysis was done with the current gold-standard methodology, pyrosequencing [9]. Due to the well-known pre-analytical issues associated with FFPE tissues, *MGMT* methylation was conclusive in just 67.8% of cases. We observed that *MGMT* methylation was significantly higher in IDH^{MUT} when compared to IDH^{WT}. These findings are in line with frequencies reported in the GBMs IDH^{WT} and IDH^{MUT} subgroup [30, 31].

We further assessed *MGMT* mRNA expression levels by NanoString, and unlike the results obtained for methylation, the assay was conclusive for practically all cases (95%). The NanoString is a well-described robust technique that does not require nucleic acid amplification or enzymatic reactions and is a valuable tool for prognostic biomarkers in FFPE samples [16, 32]. As expected, *MGMT* mRNA expression was inversely associated with *MGMT* promoter methylation. Uno and collaborators evaluated the *MGMT* promoter methylation by MSP and pyrosequencing and *MGMT* mRNA levels by real-time PCR in 51 GBMs and found an association between *MGMT* promoter methylation and low levels of *MGMT* gene expression [33].

We then evaluated the clinical and molecular features associated with the patient outcome. Analyzing just the IDH^{WT} or "de novo" GBM subgroup, which represent the majority and the most aggressive GBMs, we observed that six or more cycles of TMZ were independently associated with better patient prognosis; which is in agreement with previous studies reporting that prolonged administration increased patients' survival [34–36]. The impact of this continuation is controversial; therefore, there is a need for further studies.

Molecularly, MGMT methylation was significantly associated with patients' higher overall survival, corroborating MGMT methylation's predictive role for TMZ response in GBMs [13, 29]. Importantly, we also observed that low *MGMT* mRNA expression was independently associated with GBM higher overall survival. These findings are in accordance with other studies that reported that low levels of *MGMT* mRNA and/or low protein expression was associated with improved chemotherapy response in GBM [37–39].

We further integrated both *MGMT* methylation and mRNA levels and observed that GBMs with *MGMT* methylated and lower mRNA expression showed better outcomes compared with the combined methylated/high expression group, unmethylated/low expression group, and unmethylated/high expression group. Another study also performed the co-analysis of *MGMT* methylation status (MSP) and mRNA expression (Real-Time PCR) in 53 GBMs and showed similar results [38].

Our study exhibits some limitations, such as the relatively limited number of cases evaluated and the single institution nature of the study. Therefore, future extension and validation in other cohorts is necessary for a robust consolidation of *MGMT* expression by nCounter, and its putative inclusion in a routine setting. Additionally, the use of NanoString for only *MGMT* evaluation is not cost-effective, however, its association with other genes in order to identify gene signatures clinically relevant for molecular classification, as it is used for medulloblastomas [40], for prognostication, or for TMZ response, can lead to its implementation in a clinical setting.

The prediction of TMZ response in GBM is complex, and other factors besides MGMT are involved, such as DNA repair enzymes such as DHC2 [41] MLH1, MSH2, MSH6, and PMS2 [29] 42 and APNG [38, 43]. Therefore, further studies interrogating all these factors are warranted to have a comprehensive understanding and discovery of TMZ predictive biomarkers.

In conclusion, we found that the *MGMT* mRNA analysis using the nanostring platform can be useful as a predictor of TMZ response in GBM IDH^{WT}. Moreover, a combined analysis of both *MGMT* methylation status and mRNA expression may provide a more accurate prediction of response to TMZ for better clinical management of GBM patients.

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Author contributions IG developed the project, performed experiments, interpreted experiments, analyzed data, prepared the manuscript and figures; DM performed experiments, interpreted experiments, analyzed data, prepared the manuscript and figures; MBR performed experiments, interpreted experiments and analyzed data. LSS performed Bioinformatics' analysis and edited the manuscript. LFL performed experiments and edited the manuscript. GMG and CAP interpreted clinical data and response to treatment. MAO performed the statistical analysis. MMM provided neuropathological diagnoses and IHQ analysis. RMR designed, analyzed data, coordinated the project, and edited the manuscript. All authors read and approved the final manuscript.

Availability of data and materials The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest The authors disclose no conflicts of interest.

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