Preclinical Development

O(6)-Methylguanine-DNA Methyltransferase Is a Novel Negative Effector of Invasion in Glioblastoma Multiforme

Manik Chahal1, Bassam Abdulkarim1,3, Yaoxian Xu1,3, Marie-Christine Guiot4, Jacob C. Easaw2, Nicolas Stifani3, and Siham Sabri1,3

Abstract

The dismal prognosis of glioblastoma multiforme (GBM) is mostly due to the high propensity of GBM tumor cells to invade. We reported an inverse relationship between GBM angiogenicity and expression of the DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT), which has been extensively characterized for its role in resistance to alkylating agents used in GBM treatment. In the present study, given the major role of angiogenesis and invasion in GBM aggressiveness, we aimed to investigate the relationship between MGMT expression and GBM invasion. Stable overexpression of MGMT in the U87MG cell line significantly decreased invasion, altered expression of invasion-related genes, decreased expression of α5β1 integrin and focal adhesion kinase, and reduced their spindle-shaped morphology and migration with the empty vector control. Conversely, short hairpin RNA-mediated stable knockdown of MGMT or its pharmacologic depletion in the MGMT-positive T98G cell line were required for increased invasion. The inverse relationship between MGMT and invasion was further validated in primary GBM patient-derived cell lines. Using paraffin-embedded tumors from patients with newly diagnosed GBM (n = 59), tumor MGMT promoter hypermethylation (MGMT+ gene silencing) was significantly associated with increased immunohistochemical expression of the proinvasive matricellular protein secreted protein acidic and rich in cysteine (SPARC; P = 0.039, χ2 test). Taken together, our findings highlight for the first time the role of MGMT as a negative effector of GBM invasion. Future studies are warranted to elucidate the role of SPARC in the molecular mechanisms underlying the inverse relationship between MGMT and GBM invasion and the potential use of MGMT and SPARC as biomarkers of GBM invasion. Mol Cancer Ther; 11(11); 1–11. ©2012 AACR.

Introduction

The lethality of glioblastoma multiforme (GBM) stems from its pronounced infiltrative potential, as cells can diffusely invade beyond the margin of therapeutic resection (1). Consequently, the prognosis of GBM remains poor, with a median survival of only 15 months following standard of care therapy involving surgery, radiotherapy, and chemotherapy with the alkylating agent temozolomide (TMZ; 2). Although the putative invasive process (i.e., detachment from the primary tumor site, receptor-mediated adhesion to the extracellular matrix (ECM), degradation of the ECM, and morphologic alterations) is well characterized (3), the mechanisms by which cells instigate this invasive behavior are still under scrutiny. Therapeutic advances for GBM require a detailed understanding of the primary mediators of this behavior, as regulators of invasion may play a role in determining patient survival (4). Although potential regulators such as the p75 neurotrophin receptor (5), doublecortex, semaphorin 3B, secreted protein acidic and rich in cysteine (SPARC; 4), CAAT/enhancer binding protein β (C/EBPβ), and STAT3 (6) have been elucidated, their applicability as clinical biomarkers or anti-invasive therapeutic targets has not been conclusively determined.

Currently, there are 3 commonly used biomarkers for patients with brain tumors: isocitrate dehydrogenase 1 (IDH1) mutation, 1p/19q codeletion, and most notably, promoter methylation of the gene encoding for the DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT; ref. 7). Tumoral expression of MGMT is proposed to mediate resistance to TMZ-induced cytotoxicity via alkyl transfer at the O6 position of guanine (8). Accordingly, correlative studies show that patients with tumors displaying MGMT promoter hypermethylation or low expression of MGMT protein [i.e., MGMT(−)] are more...
likely to benefit from TMZ treatment, compared with patients with tumors displaying unmethylated MGMT, or high MGMT expression [i.e., MGMT(+); refs. 9, 10]. These studies suggest that MGMT status could be used as a predictive marker for response to alkylating agents.

The association between MGMT promoter methylation and a hypermutator phenotype was highlighted in a comprehensive genomic characterization of GBM tumors (11). These alterations could influence functional pathways dictating tumor phenotype, including invasive behavior. A potential relationship between MGMT status and tumor invasion was described in invasive Crooke’s cell adenomas, which have decreased expression of MGMT compared with noninvasive ordinary-type adenomas of Cushing’s disease (12), and in gastric carcinoma, in which MGMT promoter methylation was associated with lymph node invasion (13). In the context of GBM, Brandes and colleagues (14) recently observed that following treatment, GBM tumors with MGMT promoter methylation recurred at more distal sites from the initial radiation field. Although these studies suggest a potential relationship between MGMT expression and invasion, thus far, the effect of MGMT expression on GBM invasiveness has not been explicitly investigated.

We have previously characterized a novel inverse relationship between MGMT expression and GBM angiogenicity (15). Tumor angiogenesis and invasion, the major hallmarks of GBM aggressiveness share interdependent molecular mechanisms of regulation (16–18). We investigated the relationship between expression of MGMT protein and GBM invasiveness using isogenic overexpression and knockdown, pharmacologic depletion of MGMT in GBM cell lines, and validation in primary patient-derived GBM cell lines. Because of the prominent role of the matricellular protein SPARC in GBM invasion in vitro and in vivo (19, 20), we further investigated the relationship between tumor MGMT promoter methylation, its immunohistochemical expression, and SPARC expression in primary surgical biopsies of patients with GBM.

Materials and Methods

Cell culture and drug treatment

GBM cell lines U251, A172, U373, U138, LN18, T98G (American Type Culture Collection), U87-MG empty vector (U87/EV), and its MGMT-transfected derivative U87/MGMT (21) cells were maintained at 37°C, 95% humidified air, and 5% CO2 in Dulbecco’s Modified Eagle’s Medium (DMEM/F12 supplemented with 10% FCS and 1% penicillin/streptomycin (Invitrogen). Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (Invitrogen).

Patient-derived primary GBM cell lines originated from enzymatic dissociation of diagnostic biopsy specimens from GBM patients (kindly provided by Dr. Kenneth Petruk, University of Alberta, Edmonton, Alberta, Canada, and Dr. Joan Turner, Cross Cancer Institute, Edmonton, Alberta, Canada). Primary cell lines were maintained in DMEM/F12 supplemented with 10% FCS and 1% glutamate (Invitrogen) and were used at the following passages: 10 cell lines less than passage 10, 4 cell lines less than passage 18. All cell lines were tested for mycoplasma contamination.

O6-benzylguanine (O6BG, Sigma) was dissolved in dimethyl sulfoxide (DMSO). For depletion of MGMT using O6BG, cells were treated daily for 6 days with 10 μmol/L O6BG or DMSO as a control.

Microscopy, immunofluorescence staining, and morphometric analysis

Bright field images were taken on a Zeiss Axiosvert 200M microscope attached to a Seniscam camera using a Zeiss Plan-NEOFLUAR 10 × 0.3 or 5 × 0.15 lens.

For immunofluorescence analysis, cells were grown on coverslips coated with 8 μg/cm2 of type I collagen (collagen-I) or poly-L lysine (PLL). Cells were then fixed in 3.7% formaldehyde, permeabilized with 0.1% TritonX-100, and stained for filamentous (F)-actin using Alexa Fluor 555 phalloidin-TRITC (Invitrogen), or for focal adhesion sites using anti-FAK(pY397; BD Biosciences) followed by incubation with Alexa Fluor 488 goat anti-mouse IgG (Invitrogen). The nucleus was visualized with 4’, 6-diamidino-2-phenylindole (DAPI; Invitrogen). Images were collected on a Zeiss LSM 710/ConfoCor Observer.Z1 microscope with a Zeiss Plan-APochromat 40 × 1.3 differential interference contrast oil immersion lens.

Morphology was analyzed using Integrated Morphometry Analysis in MetaMorph 7.7 imaging software. Cell area and shape factor (resemblance to a circle) were assessed, and morphology was determined by dividing cell area by shape factor. High range values indicate a more spindle-shaped morphology.

Western blot analysis

Western blotting was conducted as described previously (15). Membranes were probed for MGMT (clone MT5.1 CD29/integrin β3, FAK(pY397; all from BD Biosciences), FAK (Upstate), integrin α6 (Cell Signaling), or β-actin (Sigma-Aldrich). Densitometric analysis (Adobe Photoshop CS3) shows relative band intensities normalized to levels of β-actin.

Cell motility assay

Nondirectional cell motility was analyzed by 2-dimensional time-lapse microscopy. Cells were seeded at 75,000 cells/well in 6-well plates coated with collagen-I (8 μg/cm2) and allowed to adhere for 45 minutes before imaging. Differential interference contrast images were acquired every minute over a 3-hour period in 2 representative fields using a Zeiss Axiosvert 200M microscope attached to a CoolSnap camera with a Zeiss Plan-NEOFLUAR ×10/0.3 lens. Composite videos were then constructed and assessed using the Track Spot Over Time function of BitPlane Imaris ×64 software.

Invasion assay

In vitro cell invasion was measured using BD BioCoat Matrigel invasion chambers (8 μm pores; BD Biosciences) following manufacturer’s instructions. Cells (25,000) were serum-starved for 24 hours, seeded in top chambers with...
DMEM, and allowed to invade toward a chemoattractant (DMEM + 10% FCS) for 24 hours. DMSO- or O6BG-treated cells were subjected to invasion assays on day 6 in the presence of DMSO or O6BG for 24 hours. Membranes were fixed with 3.7% formaldehyde and stained with 1% crystal violet. Invasive cells were visualized by bright-field microscopy using a Zeiss Axioskop2 Plus microscope attached to an Axiocam color camera with a Zeiss FLUAR 5×/0.25 lens and counted using Meta-Morph 7.7 software.

**Generation of short hairpin RNA constructs and stable MGMT shRNA transfection**

MGMT 29-mer short hairpin RNA (shRNA; 3 constructs) and control shRNAs were subcloned into the pGFP-V-RS vector (OriGene) at EcoRI and HindIII sites. The shRNA-empty vector plasmid contains a nontargeting sequence. The MGMT subclone containing the 29-mer shRNA sequence (only antisense strands indicated: 5'-GGA-CAGGATTGTGAAATGAAACGCACCA-3') was used for further experiments.

T98G cells were seeded into 6-well plates and transfected with Lipofectamine 2000 (Invitrogen) following manufacturer’s protocol. Stable MGMT shRNA clones were generated by puromycin selection (2 mg/mL for 2 weeks) and subclones were selected on the basis of GFP-positivity in single cells. T98shC1.1 was derived from T98shC1 following further exposure to puromycin. Clonogenic survival assay was used to test TMZ sensitivity (15).

**Patients’ samples and MGMT promoter methylation**

Tumor tissue paraffin blocks were collected from GBM patients diagnosed and centrally reviewed in a single institution. Ethical approval was obtained according to institutional guidelines (2006, Tom Baker Cancer Center, Calgary, Alberta, Canada). Only patients with newly diagnosed gliomas (World Health Organization grade IV) were evaluated (n = 78). MGMT promoter methylation status in GBM tumors was characterized by methylation-specific PCR (MS-PCR; 9, 22).

**Immunohistochemical staining for MGMT and SPARC**

Tissue microarray (TMA) sections (3 cores) were immunohistochemically stained for MGMT or SPARC on BenchMark XT (Ventana Medical Systems) using the technical protocol XT ultraView DAB v3. Antigen retrieval for MGMT or SPARC used an extended CC2 protocol or standard CC1 protocol, respectively (Ventana Medical Systems). Incubation with anti-MGMT antibody (1:50 for 2 hours, clone MT3;1; Millipore) or anti-SPARC (1:20,000 for 60 minutes; AON-5031, HemaTologic Technologies) was followed by incubation with UltraView horseradish peroxidase-conjugated antibody. Antigen detection was carried out using UltraView diaminobenzidine chromogen (Ventana Medical Systems). Primary antibody was omitted in the negative control. Endothelial cells served as positive internal control for MGMT and SPARC. Immunohistochemistry (IHC) staining was assessed and scored by a neuropathologist (M.-C. Guiot), who was blinded for MGMT promoter methylation status. Sections were digitalized using an Aperio scanner scope XT.

**Statistical analysis**

Data are reported as mean ± SEM and are representative of at least 3 independent experiments. Student t test was used to compare between sets of data for cell lines. Spearman rho correlation coefficients (r values) were calculated with SAS software to determine correlations between MGMT expression, invasion, and integrin expression. Correlation is significant at the 0.05 level (2-tailed). The relationship between MGMT and SPARC was assessed using χ² test. P values <0.05 denote statistical significance.

**Results**

GBM MGMT(+) cells are less invasive than MGMT (−) cells

Our previous study showed MGMT expression inversely correlated with angiogenesis (15). Because angiogenesis and invasion are critical in glioma aggressiveness and share common molecular effectors (16, 17), we sought to determine whether MGMT expression affects GBM invasive potential. Using the Gene Ontology (GO) Consortium (23) to analyze a previously conducted cDNA microarray, gene expression profiling (GEP) of U87/EV and U87/MGMT cells revealed several functional pathways were differentially regulated (15). Interestingly, biologic processes involved in invasion were significantly differentially modulated (Table 1). To assess the relationship between MGMT and GBM invasiveness, we investigated in vitro invasion of a panel of 6 established cell lines, as well as the MGMT(−) cell line U87MG stably transfected with a vector encoding for MGMT (U87/MGMT) or the control empty vector (U87/EV; 15, 21). Western blot analysis of MGMT showed that MGMT protein expression was undetectable in U87/EV, A172, U251 cell lines, and low in the cell line U373MG [24; 10% expression; i.e., MGMT(−)]. The MGMT(+) cell lines include U138MG, (intermediate level of MGMT, 40% expression), U87/MGMT, T98G, and LN18, which exhibit comparable levels of MGMT protein (Fig. 1A).

Using the Matrigel invasion assay, we established that MGMT(+) cell lines exhibited significantly less invasion compared with MGMT(−) cell lines (P < 0.001). Remarkably, the invasive potential of U87/MGMT was dramatically decreased compared with U87/EV cells (P < 0.001; Fig. 1B). Furthermore, correlation coefficient analysis showed a significant negative correlation (r = −0.83, P = 0.011) between MGMT expression and invasiveness of GBM cell lines.

β1 integrin, a regulator of glioma invasion, interacts with ECM components of the perivascular basal lamina (25) and mediates intracellular signaling pathways.
controlling cytoskeletal organization and cell movement. Western blot analysis of \( \beta_1 \) integrin and one of its binding partners, the \( \alpha_5 \) subunit revealed a different pattern of expression in MGMT(+) and (−) cells. Notably, compared with U87/EV, U87/MGMT cells displayed doublet bands reminiscent of a differential glycosylation process (26). Interestingly, there was a consistent trend of low total expression levels for both \( \beta_1 \) and \( \alpha_5 \) subunits in MGMT(+) cells (Fig. 1C) and a negative correlation between expression of MGMT and \( \alpha_5 \) (\( r = -0.805, P = 0.016 \)) or \( \beta_1 \) integrin subunits (\( r = -0.903, P = 0.002 \)). Hence, expression of MGMT is significantly negatively correlated with invasion of GBM cell lines, which is in accordance with the alteration of the pattern of \( \alpha_5\beta_1 \) integrin expression.

**Overexpression of MGMT significantly decreased migration, spindle-shaped morphology, and expression of focal adhesion kinase**

Next, we determined whether alterations in genes involved in migration (Table 1) translated to a functional modification of productive cell motility. We used 2-dimensional time-lapse video microscopy to assess nondirectional cell migration on type I collagen (collagen-I), an ECM component found in perivascular regions of the brain (27) where GBM invasion occurs (1). Compared with PLL or collagen-I. As shown by rhodamine-labeled phalloidin staining, adhesion of U87/EV cells for 1 hour induced a spindle-shaped morphology (30) characterized by irregular lamellipodia and filopodia projections on PLL and increased spreading with evidence of clear actin stress fibers on collagen-I. In sharp contrast, U87/MGMT cells retained their round appearance regardless of substrate (Fig. 2B, left panel). In addition, quantitative evaluation of morphology revealed that U87/EV cells had a more spindle-shaped appearance compared with U87/MGMT cells when plated on PLL (\( P < 0.001 \)) or collagen-I (\( P < 0.001 \); Fig. 2B, right panel), which was maintained at 24 hours and 48 hours (Supplementary Fig. S1).

Focal adhesion kinase (FAK) is a primary mediator of the molecular link between ECM-bound integrins and the cell cytoskeleton, and thus is an important regulator of cell spreading, migration, and invasion (31). GEP analysis revealed that the expression of the protein tyrosine kinase 2 gene (PTK2) encoding FAK mRNA was 2.25-fold lower in U87/MGMT cells (\( P = 0.007 \)). Accordingly, immunoblotting showed that regardless of substrate, the expression of total FAK protein and FAK(pY397) were decreased in U87/MGMT compared with U87/EV cells at both 1 hour and 24 hours after plating (Fig. 2C). Furthermore, immunofluorescence staining of FAK(pY397) showed prominent focal adhesion sites along the elongated lamellipodia of U87/EV cells, but not in U87/MGMT cells plated on collagen-I (Fig. 2D).

Thus, our results show that overexpression of MGMT correlated with profound morphologic alterations, differential cytoskeletal F-actin reorganization, and focal adhesion turnover, which may account for the reduced migratory and invasive phenotype in U87/MGMT compared with U87/EV cells.

### Table 1. Differential regulation of Gene Ontology biologic processes and KEGG pathways involved in invasion

<table>
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<th>Term</th>
<th>Count</th>
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<tr>
<td>GO:0009611 ~ response to wounding</td>
<td>61</td>
<td>2.72%</td>
<td>5.33E-03</td>
</tr>
<tr>
<td>GO:0007155 ~ cell adhesion</td>
<td>114</td>
<td>5.08%</td>
<td>2.27E-05</td>
</tr>
</tbody>
</table>

**KEGG pathways involved in invasion**

- hsa04512 ~ ECM-receptor interaction: 19 0.85% 1.49E-02
- hsa04510 ~ focal adhesion: 36 1.60% 1.49E-02

**NOTE:** Gene Expression Omnibus accession no. GSE40776. Abbreviation: KEGG, Kyoto Encyclopedia of Genes and Genomes.
Depletion of MGMT is associated with increased GBM invasiveness

To establish proof-of-concept that MGMT expression influences invasion in GBM cells, we used stable shRNA-mediated knockdown of endogenous MGMT in T98G cells. Western blot analysis showed similar levels of MGMT protein in T98G cells stably transfected with empty vector (U87/EV) or MGMT (U87/MGMT). B, histogram shows the number of invading MGMT(−) compared with MGMT(+) cells assessed by Matrigel invasion assay (mean ± SEM; n = 3). **p < 0.001. Representative photomicrographs (inset) illustrate decreased invasion of U87/MGMT compared with U87/EV cells. Scale bar, 300 μm. C, immunoblots of the same panel of GBM cell lines shows expression of β1 and α5 integrin subunits. Note that overexpression of MGMT decreased invasion and reduced expression of α5β1 integrin in U87/MGMT compared with U87/EV cells.

MGMT(+) primary patient-derived GBM cells display an invasive phenotype compared with MGMT(−) cells

To validate our findings in isogenic overexpression and knockdown models and determine their potential clinical validity, we examined invasion of 14 primary patient-derived GBM cell lines. Western blotting revealed that 9 out of 14 cell lines were MGMT(+), whereas the remaining 5 cell lines were MGMT(−) (Fig. 4A). Overall, the MGMT(−) cell lines were significantly more invasive compared with the MGMT(+) cell lines (P = 0.048), and notably, 3 of the 5 MGMT(−) cell lines displayed levels of invasion comparable to U87/EV. Interestingly, 6 of the 9 MGMT(+) cell lines exhibited a similar invasive profile as U87/MGMT, although the remaining 3 were intermediately invasive (Fig. 4B).

We further investigated whether depletion of MGMT protein was associated with increased invasiveness in primary cells. In the absence of endogenous MGMT protein, O6BG treatment of P-GM1 cells (10 μmol/L for 6 days) did not significantly alter invasion. Depletion of MGMT by O6BG by 80% in P-GM7 and in P-GM13 cells was associated with a significant increase in invasion compared with DMSO control (1.85-fold, P = 0.018 and 2.46-fold, P < 0.001, respectively). Conversely, depletion alterations translated into increased invasiveness only in the T98shC1.1 cell line (compared with T98/EV, P = 0.046; compared with T98G, P = 0.008; Fig. 3B).

The stark difference between invasion of either U87/MGMT and U87/EV (Fig. 1B) or T98/EV and T98shC1.1 cell lines (Fig. 3B), which had no detectable levels of MGMT protein, suggests that MGMT shRNA-induced knockdown in T98shC1 and T98shC8 cell lines was not sufficient to induce a shift in their invasion. Therefore, we further depleted MGMT using O6BG, a pseudosubstrate of MGMT that induces its degradation (32). Compared with their respective DMSO control conditions, treatment with O6BG (10 μmol/L for 6 days) decreased MGMT expression by 80% in both T98G and T98/EV cells (Fig. 3C). This depletion was associated with acquisition of spindle-shaped morphology in both cell lines (Supplementary Fig. S2B). When compared with DMSO control, treatment with O6BG further depleted MGMT protein by 20% and 30% in T98shC1 and T98shC8 cell lines, respectively, thereby depleting the total amount of MGMT protein by 80% (Fig. 3C). Remarkably, although O6BG treatment did not affect invasion of U87/EV cell line in the absence of endogenous MGMT, invasion was significantly increased by a fold change of 2.72 for T98G cells (P = 0.027), 2.07 for T98/EV cells (P = 0.026), 3.63 for T98shC1 cells (P = 0.017), and 2.06 for T98shC8 cells (P = 0.019) treated with O6BG compared with respective DMSO controls (Fig. 3D). Thus, as shown by shRNA-mediated knockdown of MGMT (T98shC1.1) and depletion by O6BG treatment, decreased MGMT expression by 80% seems to be a prerequisite for increased in vitro invasiveness of GBM cell lines.
of MGMT by only 60% in O6BG-treated P-GM5 cells (Fig. 4C) did not induce a significant increase in invasion (Fig. 4D). These findings corroborate that drastic decrease of MGMT expression is associated with increased in vitro invasion of established and primary patient-derived GBM cell lines.

**Relationship between MGMT status and expression of SPARC in GBM patients**

To substantiate the clinical relevance of our findings, we sought to investigate the relationship between MGMT and the expression of SPARC, a well-known proinvasive molecule (33) in a series of tumor biopsies from newly diagnosed GBM patients with no prior history of radiotherapy or chemotherapy (n = 78). Because the clinical value of immunohistochemical detection of MGMT protein is still controversial, we first investigated the correlation between MGMT expression and SPARC using analysis of MGMT promoter methylation by MS-PCR, which has been prospectively carried out in formalin-fixed paraffin-embedded (FFPE) tumors form this cohort of patients. MS-PCR results were not available in a total of 14 cases (insufficient tissue for analysis or technically unable to obtain results). Patients were dichotomized as methylated or unmethylated. MGMT promoter was found methylated in 58% (37/64) of cases, in accordance with previous reports [34%–68%, with a mean of 46% (34)].

**Figure 2.** Overexpression of MGMT in U87MG cells decreased the migratory phenotype, expression, and activation of FAK. A, left, high-magnification images of U87/EV and U87/MGMT cells migrating on collagen-I and recorded by time-lapse video. Right, histograms show average migration speed and displacement in U87/EV and U87/MGMT cells (>50 cells). B, left, U87/EV and U87/MGMT cells plated on collagen-I or PLL for 1 hour were stained for F-actin (red) and nuclear DNA (blue). Right, histogram shows quantitative evaluation of morphology as determined by cell area and shape factor (>100 cells; means ± SEM; n = 3); ***, P < 0.001. C, Western blotting showing FAK (Y397) and total-FAK levels normalized to actin in U87/EV and U87/MGMT cells plated on collagen-I or PLL for 1 hour and 24 hours. D, immunofluorescence of FAK (pY397; green) and nuclear DNA (blue) in U87/EV and U87/MGMT cells plated on collagen-I or PLL for 1 hour. Note that overexpression of MGMT (U87/MGMT) decreased migration, the spindle-shaped appearance, activation, and expression of FAK and the number of focal adhesion sites compared with U87/EV cells. Scale bars, 100 μm.
For the purpose of this study, we assessed immunohistochemical expression of MGMT using TMA sections of the same series of GBM patients ($n = 78$). Twenty-five of 78 cases (32%) were scored negative (0) or showed a heterogeneous tumor staining (1+ and 2+), whereas 53 tumor samples (68%) showed homogeneous nuclear MGMT immunostaining (≥90% MGMT(+) tumor cells: 3+; Fig. 5A–F).

As previously reported, we observed a concordance between MGMT expression and MGMT promoter methylation status in 56% of analyzed samples (35), although the subgroup of patients with unmethylated MGMT promoter displayed a strong concordance with MGMT-immunopositivity (74%).

IHC staining of SPARC showed cytoplasmic localization in GBM tumor cells. On the basis of the percentage of cytoplasmic positive tumor cells, of the 70 GBM cases with available data (insufficient tissue on TMA sections, $n = 8$), 9 were scored as negative to weakly positive (13%, score = 0 or 1), 17 were moderately positive (24%, score = 2), and 44 were strongly positive (63%, score = 3; Fig. 5G–N).

Strikingly, MGMT promoter methylation was significantly associated with increased immunohistochemical expression of SPARC (score = 3 vs. score = 0, 1, and 2; $n = 59$ cases with available data for both MGMT status and SPARC by IHC). Up to 76% of cases (25 out of 33) with MGMT promoter methylation were strongly positive for SPARC ($P = 0.039$, $\chi^2$ test). In contrast, immunopositivity of MGMT failed to correlate with SPARC expression by IHC staining ($P = 0.405$).

**Discussion**

In the present study, we identified a novel role for MGMT protein as a potential negative molecular and phenotypic regulator of GBM invasion, the main cause of treatment failure for patients with GBM. Using established and isogenic GBM cell lines differing in MGMT protein expression, primary GBM cell lines, and archived FFPE tumors from GBM patients, we provide the first direct evidence of an inverse relationship between MGMT expression and GBM invasiveness.

In particular, stable overexpression of MGMT decreased invasiveness of U87/MGMT cells compared with their counterpart and induced profound alterations of genetic, molecular, and phenotypic features. First, GEP analysis revealed modulation of a plethora of key genes encoding structural and signaling proteins involved in cytoskeleton remodeling, cell adhesion, and movement. Second, molecular determinants which may account for decreased invasion could be related to (i) a differential integrin profile, i.e., decreased total expression levels and presumably alterations of the maturation process of $\alpha_{v}\beta_{3}$ integrin, which may reduce cell spreading and migration (26) and (ii) decreased expression and activation of FAK. Owing to the role of FAK in cell motility, we selected FAK for in-depth quantitative studies and validated decreased total expression of FAK shown by GEP analysis. In accordance with decreased FAK-pTyr397 by Western blotting, immunofluorescence showed a reduced number of focal adhesions sites in U87/MGMT. Third, morphologic changes were evidenced by (i) a differential cytoskeletal F-actin reorganization and cell spreading on PLL and collagen-I substrates and (ii) motility reminiscent of a mesenchymal mode of migration for U87 cells (36), while quantitative morphometric analysis and monitoring cell migration by time-lapse showed an amoeboid motility for U87/MGMT cells. Failure to extend long polarized pseudopodia and decreased tyrosine phosphorylation of FAK in
U87/MGMT agrees with studies showing the requirement for regulated focal adhesion turnover for mesenchymal motility (37). Understanding how MGMT affects the dynamics of actin cytoskeleton turnover and elicits an amoeboid mode of migration deserves further validation on 3-dimensional substrates and may ultimately identify new targets to efficiently reduce invasiveness in vivo.

Overall, forced expression of MGMT provided some mechanistic insights into potential concerted effectors leading to decreased invasiveness. Conversely, shRNA-mediated knockdown of endogenous MGMT by 90% was required for increased invasion of T98shC1.1 cell line. In addition, drastic depletion of MGMT protein by O6BG in T98G, as well as T98shC1 and T98shC8 cell lines was required for their increase invasiveness compared with parental untreated cells.

Although we establish for the first time the relevance of MGMT to invasion in vitro, validation of our findings in primary GBM cells and patients archived tumors highlights their potential clinical significance. We showed for the first time an inverse relationship between MGMT and the proinvasive protein SPARC in a series of primary GBM patients. Interestingly, SPARC has been shown to promote migration and invasion through direct physical interactions with β1 integrin (38, 39) and activation of important signaling molecules for glioma cell motility, such as integrin-linked kinase and FAK (40, 41). In particular, MGMT promoter hypermethylation (which is expected to reflect low levels of MGMT protein) was significantly associated with high levels of tumoral SPARC expression. As previously reported in other studies, we found a limited concordance between...
immunopositivity of MGMT and promoter methylation (42) despite our caution in analyzing immunohistochemical expression of MGMT in patients with similar high glioma grade and without prior treatment with chemotherapy or radiotherapy (43). Potential limiting factors for the validation of immunohistochemical analysis of MGMT as a marker of GBM invasiveness could be related to the relatively small sample size in our cohort and MGMT intratumoral heterogeneity. In particular, MGMT protein expression has been shown to decrease progressively from the inner to the peripheral layer in GBM samples (44). Interestingly, tumor cells located in the brain parenchyma (45) or beyond the margin of tumor resection (residual tumor cells; 46) are more invasive than cells within the tumor core.

Our study reveals that SPARC might be involved in the molecular mechanisms underlying the inverse relationship between MGMT and GBM invasion. In a recent report, high expression of the membrane–cytoskeleton linker protein, Ezrin was correlated with loss of MGMT expression and increased invasion in esophageal cancer (47). Physical interactions of MGMT with binding partners may also account for downstream alterations of biologic processes, such as the transcription integrator CREB-binding protein CBP/p300 (48) and the Histone acetyltransferase p300 (EP300) known to regulate transcription via chromatin remodeling (Protein Interaction Network Analysis; ref. 49).

In sum, our data establish MGMT as a potential new negative effector of GBM invasion beyond its well-known role in response to alkylating agents. Future preclinical studies are warranted to investigate the inverse relationship between MGMT and SPARC, and validate the prognostic value of MGMT and SPARC as new invasion biomarkers in prospective studies testing anti-invasive therapies targeting invasive glioma cells with high levels of SPARC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: M. Chahal, B. Abdulkarim, J.C. Easaw, S. Sabri
Development of methodology: M. Chahal, Y. Xu, S. Sabri
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Chahal, B. Abdulkarim, M.-C. Guiot
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Chahal, B. Abdulkarim, M.-C. Guiot, J.C. Easaw, N. Stifani, S. Sabri
Writing, review, and/or revision of the manuscript: M. Chahal, N. Stifani, S. Sabri
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Abdulkarim, M.-C. Guiot, J.C. Easaw, N. Stifani
Study supervision: B. Abdulkarim, J.C. Easaw, S. Sabri

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Manik Chahal, Bassam Abdulkarim, Yaoxian Xu, et al.

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