High Incidence of *MGMT* and *RARβ* Promoter Methylation in Primary Glioblastomas: Association with Histopathological Characteristics, Inflammatory Mediators and Clinical Outcome

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Glioblastomas, the most frequent primary brain tumors in adults, are characterized by a highly aggressive, inflammatory and angiogenic phenotype. Methylation of CpG islands in cancer-related genes may serve as an epigenetic biomarker for glioblastoma diagnosis and prognosis. The aim of this study was to analyze the methylation status of four critical tumor-associated genes (*MGMT, RARβ, RASSF1A, CDH13*), and investigate possible links with inflammatory (interleukin (IL)-6, IL-8) and angiogenic mediators (vascular endothelial growth factor (VEGF), cyclooxygenase (COX)-2) and clinical outcome in 23 glioma samples (6 grade II astrocytomas, 17 grade IV glioblastomas). *RARβ* and *MGMT* genes were more frequently methylated in 70.58% and 58.8% of glioblastomas, respectively. *RASSF1A* and *CDH13* displayed a similar methylation frequency (23.52%) in glioblastomas. No gene methylation was observed in grade II astrocytomas. Tumor grade correlated positively with *MGMT* and *RARβ* methylation ($P = 0.005$ and $P = 0.019$, respectively) and the extent of necrosis ($P = 0.001$ and $P = 0.003$). Interestingly, the marker of chronic inflammation, IL-6, was positively associated with methylation of *MGMT* ($P = 0.004$), *RARβ* ($P = 0.002$), and *RASSF1A* ($P = 0.0081$) as well as the total number of methylated genes ($P < 0.0001$), indicating the important role of IL-6 in maintaining promoter methylation of these genes. VEGF expression correlated positively with *MGMT* and *RARβ* methylation although these relationships were of marginal significance ($P = 0.0679$ and $P = 0.0757$). Kaplan–Meier univariate survival analysis indicated an unfavorable survival period in patients with *MGMT* methylation compared with those without methylation ($P = 0.0474$). Our study highlights the implication of *MGMT* and *RARβ* methylation in the aggressive phenotype of primary glioblastomas. The association of *MGMT* methylation with clinical outcome indicates its potential prognostic value.

INTRODUCTION

Glioblastoma multiforme, the most advanced form of astrocytoma, accounts for ~60% of brain tumors in adults. Owing to the difficulties with early diagnosis of this malignancy and its rapid progression and frequent recurrence, glioblastoma (WHO [World Health Organization] grade IV) is an extremely life-threatening intracranial malignant tumor (1,2). Patients with grade IV glioblastomas have a mean survival time of about 1 year compared with patients with anaplastic gliomas (grade III), who survive for 2–3 years, and those with grade II gliomas, who survive for 10–15 years (3,4).

Epigenetic alterations in the coding regions of cancer-associated genes have been shown to be common events in the genesis and progression of tumors (5,6). In cancer cells, aberrant methylation of CpG islands located in the promoter regions of genes implicated in functions related to cell cycle, invasion, apoptosis or DNA repair is frequently linked to transcriptional silencing and gene repression (5,6). The aforementioned alterations of the epigenome also contribute the biological behavior of the tumor, and might modulate the response of tumor cells, to anticancer therapies (7).

These observations prompted us to examine the methylation pattern of four genes with critical cancer-related function in 23 primary glioblastoma multi-

forme tumors that had undergone surgical removal. The genes under investigation included the candidate tumor suppressor genes *Ras association domain family protein 1* (*RASSF1A*) and *retinoic acid receptor β* (*RARβ*), the cell adhesion–regulating gene *cadherin 13, H-cadherin (heart)* (*CDH13*), and the DNA repair gene *O-6-methylguanine-DNA methyltransferase* (*MGMT*).

RASSF1A, originally cloned and characterized as a human Ras effector homolog (8), contains two promoters and at least seven different transcripts (RASSF1A to RASSF1G) derived from alternative splicing and promoter usage (8). The protein encoded by transcript A (RASSF1A) interacts with the human DNA repair protein XPA in yeast 2-hybrid screens. Overexpression of RASSF1A stabilizes mitotic cyclins and induces prometaphase arrest (8). RASSF1A binds Ras in a guanosine triphosphate–dependent manner and serves as an effector mediating the apoptotic effects of Ras. Methylation of *RASSF1A* has been found in a high percentage of various human primary tumors (9,10), including gliomas (11).

The *RARβ* gene encodes RARβ, a member of the thyroid-steroid hormone receptor superfamily of nuclear transcriptional regulators. It binds retinoic acid, the biologically active form of vitamin A, which mediates cellular signaling during embryonic morphogenesis and cell growth and differentiation (12). *RARβ* transcript distribution displayed significant correlation with specific events during embryonic development of the mouse nervous system (13). *RARβ* has been found to be methylated in childhood intracranial ependymomas (13) and choroids plexus tumors (14). No previous studies on the effects of *RARβ* methylation in gliomas have been reported.

The gene product of *CDH13*, namely H-cadherin, is a member of the cadherin superfamily and plays a key role in cell–cell adhesion. H-cadherin is anchored to the cell-surface membrane through a glycosyl phosphatidylinositol

moiety and unlike other cadherins such as E-cadherin, N-cadherin, and P-cadherin lacks the cytoplasmic domain (15). Recent studies have demonstrated the role of *CDH13* as a tumor suppressor and invasion suppressor gene in lung, breast, colorectal, and gastric cancers (16,17). Furthermore, downregulation of H-cadherin due to hypermethylation in the promoter region of the *CDH13* gene appears to be related to the tumorigenesis and invasiveness of these cancers. However, expression of H-cadherin and the methylation status of *CDH13* in brain tissue have not been thoroughly investigated (18).

MGMT is the most investigated gene in astrocytic tumors. As a key DNA repair enzyme, MGMT specifically removes promutagenic methyl groups from the O-6 position of guanine by transferring them to cysteine acceptor sites on the protein itself (19). Promoter methylation of *MGMT* has been shown to be linked to the presence of G:C to A:T transition mutations in TP53 in many types of cancer, such as low-grade astrocytoma, anaplastic astrocytoma, and secondary glioblastomas (20,21). The validity of *MGMT* methylation as a prognostic and predictive indicator is still controversial. According to some investigators, *MGMT* promoter methylation has been associated with better survival in glioblastoma patients treated with radiotherapy and alkylating agents (20,21). In other reported studies, however, *MGMT* promoter methylation was not confirmed to be a positive predictive marker of response to chemotherapy with alkylating agents (22), and little is known about whether *MGMT* promoter methylation can be of prognostic value for glioblastoma patients not treated with chemotherapy.

Inflammatory mediators have important roles in carcinogenesis. Interleukin (IL)-6, an inflammatory cytokine that is a crucial mediator in the host immune defense response, also plays a pleiomorphic role in several kinds of human diseases including tumors. We have previously shown that IL-6 secretion and expression

levels are strongly associated with the progression and invasion of glioblastomas (23). The chemotactic cytokine IL-8 has been implicated in the progression of various malignancies including those that arise in the central nervous system and may play a role in angiogenesis (24). Given the long-recognized prominence of vasculature in high-grade astrocytic gliomas, it is not surprising that several investigators have looked into the mechanisms promoting angiogenesis in these tumors. In this context, vascular endothelial growth factor (VEGF) figures prominently as the main effector in matching vascular supply to the metabolic requirements of the tumor (25). VEGF expression is under the control of hypoxia-inducible factor (HIF)-1α, a transcription factor regulating the adoptive responses to hypoxia (25). Modulation of the HIF-1α/VEGF axis by tissue oxygen concentration is largely responsible for the promotion of angiogenesis in glioblastomas (26). Other molecules implicated in the stimulation of angiogenesis in astrocytic tumors include cyclooxygenase (COX)-2, which is induced by inflammatory signals, mitogens, cytokines and growth factors (27). Previous studies from our group (27,28) revealed the coordinate expression and topographical relationship of IL-6, IL-8, COX-2 and VEGF in the same glioma tumor areas (for example, perinecrotic areas), attesting to their intimate liaison in terms of cancer-induced angiogenesis, which is probably secondary to induction of multiple interdependent molecular pathways.

In the present study, we aimed to analyze the methylation status of four genes involved in important steps of tumorigenesis (*MGMT, RARβ, RASSF1A,* and *CDH13)* and investigate possible links with inflammatory (IL-6, IL-8) and angiogenic mediators (VEGF, COX-2) as well as histopathological characteristics (tumor grade, extent of necrosis) and clinical outcome. The identification of possible associations between cytological and genetic features may prove to be of pertinent importance for the development of efficient treatment modalities.

MATERIALS AND METHODS

Patients

Twenty-three patients with histologically diagnosed astroglial tumors randomly admitted to the 'Evangelismos' General Hospital (Athens, Greece) were enrolled in the present study. In all cases, the diagnoses and grading were peer reviewed by two experienced pathologists (G Levidou, P Korkolopoulou), according to the principles of the latest WHO classification (29). Seventeen cases were categorized as glioblastoma (grade IV) and six as diffuse astrocytoma (grade II). Informed consent was obtained from all patients and the study was approved by ethics committee of the University of Athens Medical School.

All patients had undergone tumor debulking, followed by assessment of the extent of surgery by tomography (computed tomographic scan) and magnetic resonance imaging (MRI) within 48 h (Figure 1). In each case the presence and extent of necrosis (focal/extensive) were also assessed by imaging techniques and were compared with those in tissue specimens. The mean age of patients was 58.14 years, ranging from 25 to 76 years, and the male-to-female ratio was 15 to 9. The medical records of glioma patients showed that they were under antiepileptic treatment. Three of the patients were also receiving drugs for diabetes mellitus and hyperlipidemia. Regular blood tests of patients during their hospitalization revealed no further abnormalities in their biochemical and lipid profiles.

Tissue Specimens

Fresh tumor specimens were cut immediately after removal into strips measuring approximately $1.5 \times 1.5 \times 0.5$ cm. The strips were then divided into three fragments. The central fragment, for quality control purposes, was formalin fixed and paraffin embedded. Hematoxylin and eosin sections from the central fragment were used for diagnosis and grading, and examined to ensure that the material was representative and
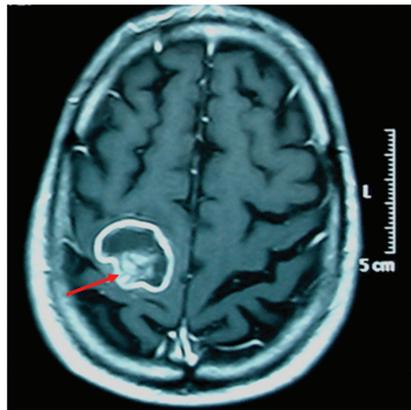
Figure 1. MRI scan, presenting a transverse section of the brain. The glioblastoma tumor (arrow) is located in the right posterior parietal lobe (image taken from 'Evangelismos' General Hospital files, Athens, Greece).

of good quality. Only samples consisting of more than 80% neoplastic tissue were employed. In each case the presence and extent of necrosis was similar to that seen in computed tomography and MRI tomography.

Immunohistochemical Analysis

Immunohistochemical localization of IL-6, IL-8, COX-2, and VEGF expression levels was performed in formalin-fixed paraffin-embedded tissue sections of all patients, as previously described (28). The following antibodies were used: anti–IL-6 (goat polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:100; anti–IL-8 (rabbit polyclonal, BioSource International, Camarillo, CA, USA) diluted 1:50; anti–COX-2 (Scl746, monoclonal, Santa Cruz Biotechnology) diluted 1:100; and anti-VEGF (clone G153-694, monoclonal; Pharmingen BD, San Diego, CA, USA) diluted 1:75. The incubation time was 1 h at room temperature for VEGF and 18 h at 4°C for IL-6, IL-8 and COX-2. The buffers, blocking solutions, secondary antibodies, avidin-biotin complex reagents and chromogen were supplied in a detection kit (LSAB detection kit; Dako, Carpinteria, CA, USA). The percentage of neoplastic cells was estimated by using light microscopy.

DNA Extraction and Bisulfite Modification

Genomic DNA from frozen tissues was extracted by NucleoSpin Tissue (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. DNA concentration was estimated by spectrophotometry. DNA (500 ng) obtained from tissue samples, or cell line DNA was chemically modified using the EpiTect Bisulfite kit (Qiagen, West Sussex, UK) according to the manufacturer's protocol. The modified DNA was used as a template for polymerase chain reaction (PCR) analysis. DNA from normal lymphocytes was used as unmethylated control. Human breast cancer cell line (*MCF-7*) was used as a positive control for methylated alleles.

Methylation-Specific PCR

The methylation status of the genes was determined by the method of methylation-specific PCR (MSP). PCR amplification was performed by using 150–200 ng of treated DNA as the template (11). Two sets of primers were used at the same position, one set specific for DNA methylated at CpG sites, and one specific for fully unmethylated DNA. PCR conditions, annealing temperatures and the expected PCR product sizes are summarized in Table 1. Products were visualized on 2% agarose gels or 6% nondenaturing polyacrylamide gels.

Statistical Analyses

For basic statistical calculations, IL-6, IL-8, VEGF, and COX-2 expression were treated as continuous variables. The association of the above parameters with grade, necrosis, and methylation status was tested with Kruskal–Wallis ANOVA or Mann–Whitney U test. The correlations among IL-6, IL-8, VEGF and COX-2 expression and gene methylation status were examined with Spearman correlation coefficient. Survival analysis was performed using death by disease as the endpoint. The effect of various parameters on clinical outcome was assessed by plotting survival curves according to the Kaplan–Meier method and comparing

Table 1. Primer sequences and PCR conditions for MSP analysis.

Primer name	5′–3′ Primer sequence forward	5′–3′ Primer sequence reverse	Genomic position[a]	AT,[b] °C	Product size, bp
CDH13-U	5′-TTGTGGGGTTTGTTTTTTGT	5′-AACATTTTCATTCATACACACA	–267	62	243
CDH13-M	5′-TCGCGGGGTTCGTTTTTCGC	5′-GACGTTTTCATTCATACACGCG	–267	62	243
MGMT-U	5′-TTTGTGTTTTGATGTTTGTAGGTTTTTGT	5′-AACTCCACACTCTTCCAAAAACAAAACA	–46	59	93
MGMT-M	5′-TTTCGACGTTCGTAGGTTTTCGC	5′-GCACTCTTCCGAAAACGAAACG	+26	59	81
RARβ-U	5′-TTAGTAGTTTGGGTAGGGTTTATT	5′-CCAAATCCTACCCCAACA	–69	59	233
RARβ-M	5′-GGTTAGTAGTTCGGGTAGGGTTTATC	5′-CCGAATCCTACCCCGACG	–71	59	235
RASSF1A-U	5′-GGTTTTGTGAGAGTGTGTTTAG	5′-CACTAACAAACACAAACCAAAC	–73	59	169
RASSF1A-M	5′-GGGTTTTGCGAGAGCGCG	5′-GCTAACAAACGCGAACCG	–73	64	169

[a]The 5′ positions of the sense unmethylated and methylated are numbered relative to the transcription start site of the gene concerned
[b]AT, annealing temperature; U, unmethylated primer; M, methylated primer.

groups using the log-rank test, as appropriate. All statistical calculations were performed using the statistical package STATA 9.0 for Windows Software. Differences were considered statistically significant when the *P*-value (two-sided) was < 0.05.

RESULTS

Frequency of Gene Promoter Methylation by MSP

In 23 primary glioblastomas the methylation status of the four gene promoter regions was determined by MSP. *RARβ* and *MGMT* genes were more frequently methylated in 70.58% and 58.8% of glioblastoma tissues, respectively. *RASSF1A* and *CDH13* exhibited a similar positive methylation profile in 23.52% of glioblastoma cases. No gene methylation was observed in grade II astrocytomas. Furthermore, *MGMT* and *RARβ* methylation correlated positively with tumor grade (Fisher exact test, *P* = 0.005 and

P = 0.019, respectively) and the extent of necrosis (Fisher exact test, *P* = 0.001 and *P* = 0.003; Table 2). The total number of methylated genes (patients with absolute number of three methylated genes) was positively correlated with the extent of necrosis (Kruskal–Wallis ANOVA, *P* = 0.0041) and marginally significant with tumor grade (Kruskal–Wallis ANOVA, *P* = 0.069).

Immunohistochemical Assessment of Inflammatory and Angiogenic Mediators

IL-6 expression was detected in 22 of 23 cases (median 30%) and specifically localized in tumor cells and macrophages as well as in areas of large ischemic necrosis, as previously described (23,28). The percentage of IL-6–positive neoplastic cells ranged from 2% to 80% (Figure 2).

IL-8 expression was recorded in all 23 cases. Positive neoplastic cells represented 1% to 40% (median value, 0.10) of the neoplastic population and displayed
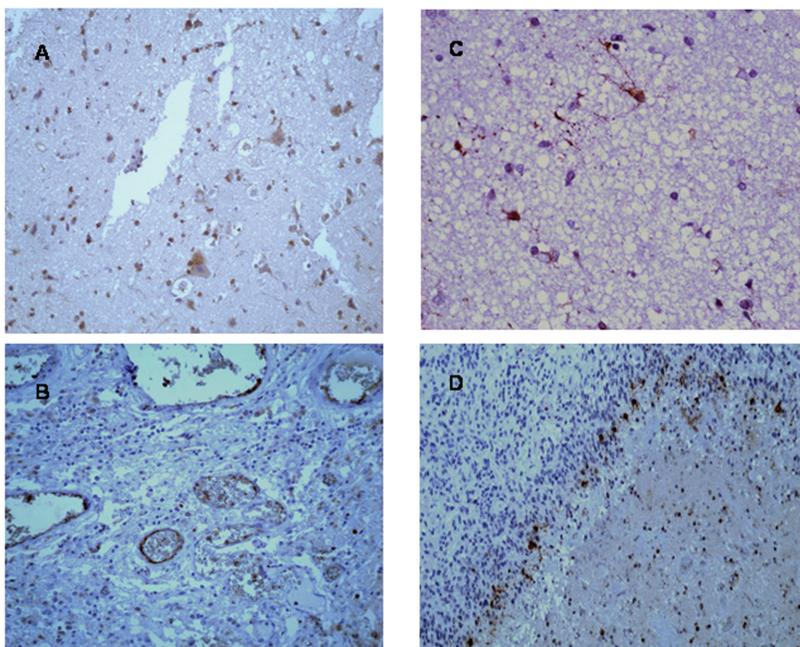
a perinecrotic distribution. Moreover, IL-8 expression was identified in endothelial cells within glomerouloid vascular structures as well as in inflammatory cells around foci of necrosis (Figure 2). The median IL-8 expression level by the neoplastic cells was significantly lower than that of IL-6 (Wilcoxon signed-rank test, *P* = 0.0006).

Importantly, a significant relationship emerged between IL-6 expression and increasing histological grade (Kruskal–Wallis ANOVA test, *P* = 0.0264) or extensive necrosis (Mann–Whitney *U* test, *P* = 0.0209). On the other hand, IL-8 expression increased with increasing histological grade and degree of necrosis, albeit the latter relationship was of marginal significance (Kruskal–Wallis ANOVA test, *P* = 0.0204 and *P* = 0.0759, respectively).

COX-2 and VEGF expression was detected in the cytoplasm of neoplastic cells of all cases, with an increased expression in grade IV compared with grade II astrocytomas (Mann–Whitney *U* test, *P* = 0.024

Table 2. Associations of *MGMT*, *RARβ*, *RASSF1A*, *CDH13* promoter methylation with histopathological characteristics.

	MGMT methylation			*RARβ* methylation			*RASSF1A* methylation			*CDH13* methylation		
	Presence, n	Absence, n	*P*	Presence, n	Absence, n	*P*	Presence, n	Absence, n	*P*	Presence, n	Absence, n	*P*
Necrosis			0.001			0.003			0.383			0.383
Absence	8	0		8	1		8	0		8	0	
Focal	0	1		0	0		1	0		1	0	
Diffuse	3	11		5	9		10	4		10	4	
Histologic grade			0.005			0.019			0.539			0.539
II	6	0		6	0		6	0		6	0	
IV	5	12		7	10		13	4		13	4	

Figure 2. Expression of inflammatory cytokines in astrocytic tumors. IL-6 immunoreactivity in the cytoplasm of neoplastic cells of grade II astrocytoma (A) and grade IV glioblastoma (B). IL-8 immunostaining in the cytoplasm of grade II neoplastic cells (C) and grade IV glioblastoma (D) (magnification 200×).
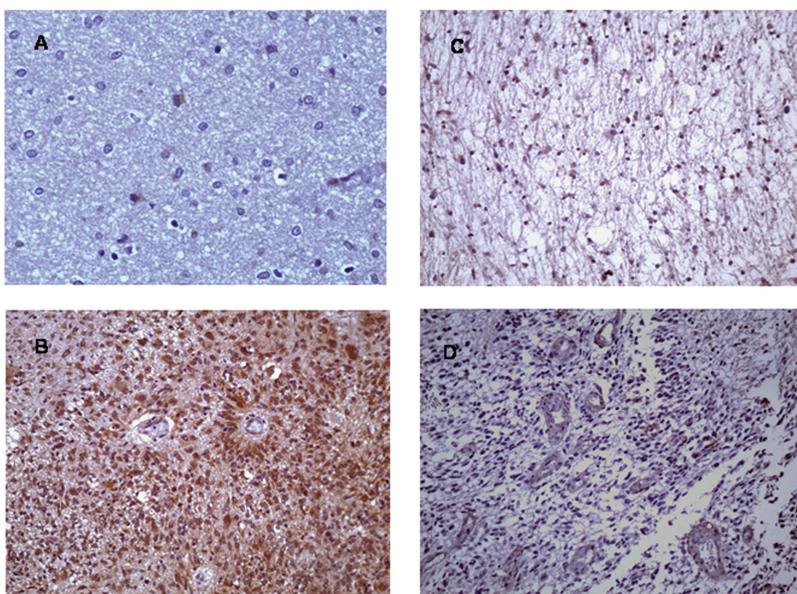
Figure 3. Expression of angiogenic factors in astrocytic tumors. VEGF immunoreactivity in neoplastic cells of grade II astrocytoma (A) and grade IV glioblastoma (B). COX-2 immunostaining in neoplastic cells of grade II astrocytoma (C) and grade IV glioblastoma (D) (magnification 200×).

and $P = 0.001$, respectively; Figure 3). A positive correlation was obtained between COX-2 and VEGF (Spearman correlation coefficient, $P < 0.0001$, $r = 0.485$). A significant positive correlation emerged between IL-6 expression on the one hand and COX-2 or VEGF on the other (Spearman correlation coefficient, $P = 0.0033$, $r = 0.5856$ and $P = 0.05$, $r = 0.4095$, respectively). The same applied to IL-8 expression (Spearman correlation coefficient, $P = 0.0079$, $r = 0.5392$ between COX-2 and IL-8 and $P < 0.001$, $r = 0.7954$ between VEGF and IL-8).

Correlation between Gene Promoter Methylation Status and Inflammatory/Angiogenic Mediators

The correlations between gene promoter methylations and inflammatory/angiogenic factors are shown in Table 3. Among the two inflammatory cytokines under investigation, only IL-6 displayed statistically significant positive correlation with *MGMT*, *RARβ*, *RASSF1A*, and *CDH13* promoter methylation (Mann–Whitney U test, $P = 0.004$, $P = 0.002$, $P = 0.0081$, respectively). A positive correlation was also obtained between the total number of methylated genes and IL-6 (Spearman correlation coefficient, $P < 0.0001$, $r = 0.80$). IL-8 expression showed no correlation with the methylation status of any gene (Mann–Whitney U test, $P > 0.10$).

Regarding the angiogenic parameters, *MGMT* and *RARβ* displayed a positive correlation with VEGF of marginal significance (Mann–Whitney U test, $P = 0.0679$, $P = 0.0757$), whereas *RASSF1A* and *CDH13* methylation did not present any significant correlation with VEGF (Mann–Whitney U test, $P = 0.8034$, $P = 0.8040$). The same applied also for all the methylated genes under investigation and COX-2 expression (Mann–Whitney U test, *MGMT*, $P = 0.6603$; *RARβ*, $P = 0.1640$; *RASSF1A*, $P = 0.2821$; *CDH13*, $P = 0.2821$).

Gene Promoter Methylation and the Impact on Prognosis

RASSF1A, *RARβ*, and *CDH13* had no impact on survival time in primary

Table 3. Correlations of *MGMT, RARβ, RASSF1A,* and *CDH13* promoter methylation with inflammatory and angiogenic factors.

	MGMT methylation			RARβ methylation			RASSF1A methylation			CDH13 methylation		
	Presence median (range)	Absence median (range)	P	Presence median (range)	Absence median (range)	P	Presence median (range)	Absence median (range)	P	Presence median (range)	Absence median (range)	P
IL-6 expression, %	64.5 (7–80)	15 (0–30)	0.004	69.5 (25–80)	15 (0–40)	0.002	75 (60–80)	25 (0–80)	0.0081	55 (25–80)	30 (0–80)	0.1320
IL-8 expression, %	17.5 (5–70)	10 (2–30)	0.2892	25 (10–70)	10 (2–30)	0.2326	17.5 (5–40)	15 (2–70)	0.2326	25 (5–70)	30 (2–30)	0.1640
VEGF expression, %	60 (50–80)	50 (40–70)	0.0679	65 (50–80)	60 (40–70)	0.0757	65 (5–80)	60 (40–70)	0.8034	65 (5–80)	60 (40–70)	0.8040
COX-2 expression, %	30 (10–70)	25 (15–40)	0.6603	37.5 (10–70)	25 (15–40)	0.1640	37.5 (10–70)	25 (15–40)	0.2821	37.5 (10–70)	25 (15–40)	0.2821

glioblastoma patients (Kaplan–Meier univariate analysis, $P = 0.6281$, $P = 0.5096$, $P = 0.8461$, respectively); that is, mean survival times were similar for patients with or without these gene defects. However, *MGMT* promoter methylation affected mean survival time of the study patients. The median survival duration of patients with *MGMT* methylation was 12 months, compared with those who had no *MGMT* methylation and survived for more than 16 months (Kaplan–Meier univariate analysis, $P = 0.0474$; Figure 4).

DISCUSSION

Methylation status has been previously reported to be a significant prognostic variable for various tumor types. In this study of four genes involved in critical stages of glioma tumorigenesis we investigated whether the methylation status of these genes is associated with histopathological characteristics and inflammatory and angiogenic parameters, as well as clinical outcome.

An interesting finding was that the genes under investigation showed no methylation in grade II astrocytomas, whereas they presented a high degree of methylation in grade IV glioblastomas. The most frequently methylated genes in our study were the DNA repair gene *MGMT* and the tumor-suppressor gene *RARβ*, which were present in 70.58% and 58.8% of glioblastoma tissues, respectively, suggesting promoter methylation of these genes to be a common event in glioma tumorigenesis. A lower but significant methylation frequency was observed for *RASSF1A* and *CDH13* genes, reaching 23.52% for both genes.

These data are in concert with a recent report of a study in which *MGMT* promoter methylation was detected in 72% of primary glioblastomas (30). In previous studies, *MGMT* promoter methylation was found in 36%–68% of primary glioblastomas (31,32), both in DNA samples isolated from frozen tumor tissue and in paraffin-embedded sections. In selected cases of long-term (more than 3 years) survivors with glioblastoma, *MGMT* promoter methylation reached 74% (33). One of the reasons for the high percentage of *MGMT* promoter methylation in our study may be a higher sensitivity of the MSP assay from cryopre-
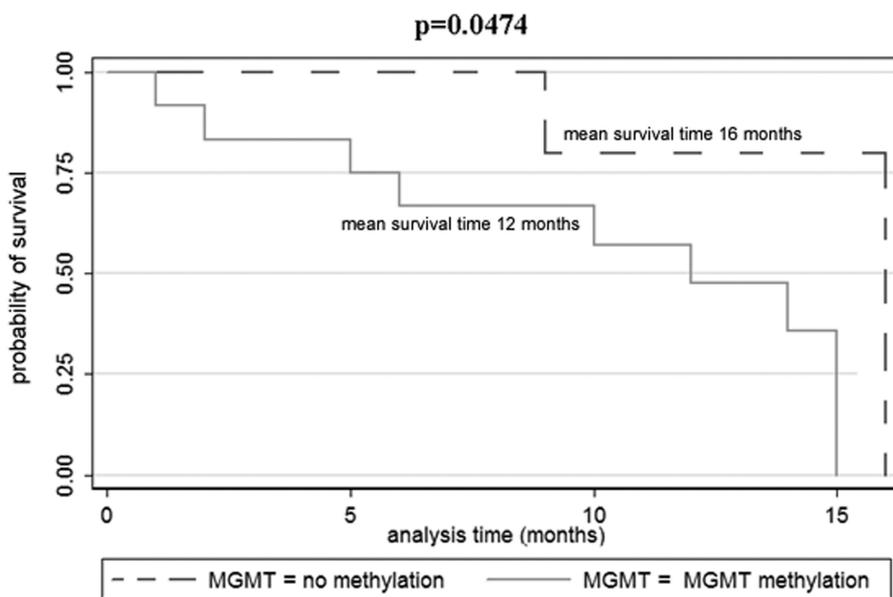
Figure 4. Correlation between *MGMT* methylation status and overall survival of glioma patients.

served tissue samples than from paraffin sections.

Active MGMT has a protective role against cytotoxic and mutagenic stress due to the removal of alkylating adducts from the O-6 position of guanine, which leads to chemotherapeutic alkylating drug resistance, whereas silencing of the *MGMT* gene has been suggested to predispose the neoplastic clones to acquisition of the guanine-to-adenine point mutations in K-*ras* and *p53* (34).

Early studies showed that human tumor cells deficient in *MGMT* are unable to demethylate O-6-methylguanine in DNA and are more sensitive to alkylating agents than cells proficient in *MGMT* (35). Brain tumors expressing low levels of MGMT are more sensitive to chemotherapy based on alkylating agents, and MGMT depletion by O-6-benzylguanine enhances the therapeutic efficacy *in vitro* and in patients (36). Loss of MGMT expression is rarely caused by deletion, mutation, or rearrangement of the *MGMT* gene or mRNA instability. Inactivation of the *MGMT* gene by promoter methylation in glioma cell lines or in many primary tumors is a common occurrence. Therefore, regulation of MGMT expression seems to be an epigenetic event directly dependent on *MGMT* promoter methylation.

In our study, the presence of *MGMT* methylation had an impact on survival, indicating that patients without *MGMT* methylation had a better mean survival time than those bearing it. The prognostic and predictive value of *MGMT* determination remains uncertain. According to some reports, *MGMT* promoter methylation has been associated with better survival in glioblastoma treated with radiotherapy and alkylating agents (20,21). However, *MGMT* promoter methylation was not confirmed by others to be a positive predictive marker of response to chemotherapy with alkylating agents (22). Few data are available to elucidate whether *MGMT* promoter methylation can be predictive for glioblastoma patients who are not treated with chemotherapy. Some studies have revealed that

MGMT promoter methylation *per se* is an independent favorable prognostic factor irrespective of treatment (22).

RARβ is the second gene found to be frequently methylated in grade IV glioblastomas (58.8%). Being a nuclear receptor, *RARβ* is often found deregulated in several tumors. In glial tumors, there is only one study in which, irrespective of the tumor histological grade, a constant decrease of RARβ expression was found in reference to normal tissue. However, *RARβ* methylation was not always associated with its transcriptional silencing (37). Additionally, hypermethylation of the *RARβ* gene has been detected in papillomas but was absent in normal choroid plexus tissue, suggesting that methylation of the *RARβ* gene could be an early event in the development of choroid plexus tumors (13,14).

The fact that both *MGMT* and *RARβ* promoter methylation correlated positively with tumor grade and the extent of necrosis in glioblastoma tissue indicates the possible pathological involvement of these genes and their implication in pathways engaged in tumor progression.

RASSF1A, a tumor suppressor gene on 3p21.3, is frequently inactivated during tumorigenesis in a variety of cancers, most commonly by promoter methylation. Several studies correlate the loss of *RASSF1A* gene expression to promoter methylation in 32% to 70% of astrocytomas (38,39). Notably, *RASSF1A* promoter methylation seems to increase with tumor grade. In our study, *RASSF1A* promoter methylation was found in 23.52% of glioblastomas and was not correlated with tumor grade or degree of necrosis, possibly owing to small sample number. However, a recent study investigating *RASSF1A* methylation in 53 astrocytomas and 10 high-grade glioma cell lines showed 92% of tumor samples being methylated for *RASSF1A*, suggesting that inactivation of this tumor suppressor gene by promoter methylation is a crucial and frequent event in astrocytoma initiation and progression (11). Furthermore, the fact that *RASSF1A* promoter methylation occurs

rarely in normal tissues, including the brain, renders its analysis a useful diagnostic tool in early tumor detection and disease monitoring.

The *CDH13* gene, which codes for the calcium-dependent protein H-cadherin, was also found methylated in 23.52% of grade IV glioblastomas. Loss of expression of *CDH13* has been found in parallel with the hypermethylated promoter CpG islands in various cancers (40). However, only one study of gliomas, in which investigation of *CDH13* in low grade astrocytomas revealed no methylation patterns, had findings in accordance with our observations (18).

An important finding of our study is the relationship between inflammatory mediators and the defective genetic profile of gliomas. A strong association exists between states of chronic inflammation and cancer, and it is believed that mediators of inflammation may be responsible for this phenomenon. It has been previously reported that chronic inflammation along with aging and viral infections may be associated with methylation (41), and much work is being done to unveil how the aberrant methylation is induced and maintained.

In our study, IL-6 expression in tumor samples was positively correlated with *MGMT*, *RARβ* and *RASSF1A* methylation, suggesting a possible mechanism employed by IL-6 in the growth and survival of gliomas. Previous investigators have described the ability of IL-6 to induce the expression and activity of DNMT-1, the maintenance methylase, in the human erythroleukemia cell line K-562 (42). These findings suggest that IL-6 may affect epigenetic mechanisms beyond its previously described functions involving the mediation of inflammation and differentiation. In K-562 cells, IL-6 induces the expression of the Fli1/ERG-B transcription factor by direct activation of STAT-3, a transcriptional regulator shown previously to possess oncogenic potential (43). The activation of STAT-3 has been shown to be important in the oncogenesis of many types of cancer, including gliomas (44).

Another study has demonstrated an important link between IL-6 signaling and p53 methylation in a human multiple myeloma cell line, suggesting that IL-6 can establish and maintain promoter methylation.

The prognostic value of IL-6 in gastric cancer is supported by data obtained through analysis of the associations of serum IL-6 levels with methylation status of *p16*, *death-associated protein kinase* (*DAPK*), *MGMT*, and *E-cadherin*. Low serum IL-6 levels were associated with *p16* or *DAPK* gene methylation in patients with this type of cancer (45).

On the other hand, although IL-6 levels seem to be crucial for maintenance of gene promoter methylation, the mechanisms regulating IL-6 expression remain unclear. Studies on the transcriptional control of IL-6 have identified at least four transcription factor binding sites located within the *IL-6* promoter region, including nuclear factor kappa B, cAMP response element-binding protein, CCAAT/enhancer binding protein, and activator protein 1 binding sites, whose roles in the regulation of *IL-6* expression are complex and dependent on cell type and alterations in microenvironments. Several studies also suggest a role for DNA methylation in the epigenetic regulation of cytokine gene expression (46).

To the best of our knowledge, this is the first study to provide evidence of an important link between IL-6 and epigenetic gene silencing in glioblastomas. Further studies are required to elucidate the actual mechanisms underlying the epigenetic control of tumor cell functions by IL-6.

In summary, our results demonstrated a role of *MGMT* and *RARβ* genes in glioma tumorigenesis, with *MGMT* promoter methylation being of prognostic value for glioblastoma patients. Furthermore, the positive correlation between IL-6 expression levels and methylation status of *MGMT*, *RARβ* and *RASSF1A* genes is one of the possible mechanisms of inflammatory mediators involved in the growth and survival of glioma tumors.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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