Epigenomic Profiling Reveals Novel and Frequent Targets of Aberrant DNA Methylation-Mediated Silencing in Malignant Glioma

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Abstract

Malignant glioma is the most common central nervous system tumor of adults and is associated with a significant degree of morbidity and mortality. Gliomas are highly invasive and respond poorly to conventional treatments. Gliomas, like other tumor types, arise from a complex and poorly understood sequence of genetic and epigenetic alterations. Epigenetic alterations leading to gene silencing, in the form of aberrant CpG island promoter hypermethylation and histone deacetylation, have not been thoroughly investigated in brain tumors, and elucidating such changes is likely to enhance our understanding of their etiology and provide new treatment options. We used a combined approach of pharmacologic inhibition of DNA methylation and histone deacetylation, coupled with expression microarrays, to identify novel targets of epigenetic silencing in glioma cell lines. From this analysis, we identified >160 genes up-regulated by 5-aza-2′-deoxycytidine and trichostatin A treatment. Further characterization of 10 of these genes, including the putative metastasis suppressor *CST6*, the apoptosis-inducer *BIK*, and *TSPYL5*, whose function is unknown, revealed that they are frequent targets of epigenetic silencing in glioma cell lines and primary tumors and suppress glioma cell growth in culture. Furthermore, we show that other members of the TSPYL gene family are epigenetically silenced in gliomas and dissect the contribution of individual DNA methyltransferases to the aberrant promoter hypermethylation events. These studies, therefore, lay the foundation for a comprehensive understanding of the full extent of epigenetic changes in gliomas and how they may be exploited for therapeutic purposes. (Cancer Res 2006; 66(15): 7490-501)

Introduction

Malignant glioma is one of the most devastating and lethal forms of human cancer despite significant efforts to understand its genetic etiology and improve treatment regimens. Gliomas are the most prevalent central nervous system tumor of adults and are thought to arise from a glial progenitor cell (1). Patients diagnosed with glioblastoma, the most common form of glioma (~65% of cases), have a median survival time of only 9 to 12 months and a 5-year survival rate of <3% despite the use of aggressive surgery, radiation, and chemotherapy (2). Additional important features of malignant glioma include its highly infiltrating nature into normal adjacent brain tissue, rendering them incurable by surgery alone, and their relative resistance to radiation and most forms of chemotherapy.

It has become clear that cancers in general arise from both genetic and epigenetic changes. Genetic alterations in malignant glioma have been extensively studied and include p53 mutations, deletion of chromosome 17p13.1, and allelic loss from a number of other genomic regions (e.g., 9p, 11p, and 13q; ref. 3). Epigenetic changes, such as promoter hypermethylation and chromatin structural changes favoring transcriptional silence (e.g., histone deacetylation), have also emerged as important contributors to tumorigenesis (4). Tumor suppressor genes may be inactivated by genetic changes, such as mutations and deletions, and/or by aberrant epigenetic changes, such as DNA methylation and histone tail modifications.

DNA methylation, mediated by a family of DNA methyltransferases (DNMT1, DNMT3A, and DNMT3B), is a potent and heritable gene silencing system that is critical for normal embryonic development but becomes deregulated in nearly all tumor cells (4). Inhibitors of DNA methylation, such as 5-aza-2′-deoxycytidine (5-azadC), induce reexpression of epigenetically silenced tumor suppressor genes and restore cell growth control or induce apoptosis (5). 5-azadC given to colon cancer-prone mice significantly reduced tumor incidence and tumor size (6) and acted as a chemopreventive agent in a murine lung cancer model (7). Histone deacetylase (HDAC) inhibitors, including trichostatin A (TSA), depsipeptide, and phenylbutyrate, induce a more limited set of epigenetically silenced genes (8); however, combining DNA methylation and HDAC inhibitors yields a synergistic effect on gene reactivation (9) and tumor suppression in a murine model of lung cancer (10). Therefore, there is great interest in using these agents, possibly in combination with conventional chemotherapeutic drugs, to treat cancer. In addition, because DNA methylation changes are early events and occur more frequently than individual genetic changes, the feasibility of developing them as diagnostic or prognostic markers of disease, through the detection of aberrantly hypermethylated tumor cell DNA shed into bodily fluids, is being very actively explored (11).

Previous studies on the role of epigenetic silencing in glioma have been carried out using a candidate gene approach and by a genome-wide screening method. The former revealed that genes, such as *TIMP3* (12), whose gene product inhibits the activity of matrix metalloproteinases, and *EMP3* (13), a myelin-related gene believed to be involved in cell proliferation and cell-cell

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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interactions and located within the commonly deleted 19q13.3 region, are frequently targeted for DNA methylation-mediated silencing in glioma. Epigenetic silencing of the *MGMT* gene, involved in the repair of DNA damage due to alkylation of the O^6 position of guanine, is also frequent in glioma (14). Silencing of MGMT by DNA methylation is associated with longer survival of newly diagnosed glioblastoma patients and is also an indicator of increased survival in patients treated with radiation plus the alkylating agent temozolomide (15). A genome-wide screen for aberrant DNA methylation events in glioma using restriction landmark genomic scanning revealed that ~1,500 CpG islands may be subject to aberrant hypermethylation in low-grade gliomas, suggesting that epigenetic alterations are widespread and likely contribute significantly to gliomagenesis (16).

Here, we have coupled pharmacologic inhibition of epigenetic modifications, by treating four glioma cell lines with 5-azadC and TSA, with microarray-based gene expression profiling, to identify targets of aberrant epigenetic silencing in malignant glioma. More than 160 genes were up-regulated by this combination of epigenetic inhibitors. In-depth analysis of 10 of these genes, including *TSPYL5, CST6, TACSTD2, TAC1, BIK*, and *CLIC3*, using bisulfite genomic sequencing (BGS) and methylation-specific PCR (MSP), reveals that they are frequently methylated in glioma cell lines and more importantly primary brain tumors. They are largely hypomethylated in normal brain tissue, strongly suggesting that the methylation is tumor specific. Hypermethylated TSPYL5 alleles were detectable in nearly 100% of the primary glioma tumors we analyzed. Furthermore, three genes (*TSPYL5, BIK*, and *TACSTD2*) exhibit consistent and marked growth suppressive properties in glioma cell lines, suggesting that they act as tumor suppressors.

Materials and Methods

Cell lines, tissue culture, and drug treatments. The glioblastoma cell lines LN-229, U-118 MG, U-87 MG, DBTRG-05MG, and LN-18 and the glioblastoma multiforme cell line T98G were purchased from the American Type Culture Collection (Manassas, VA) and maintained in McCoy's 5-a medium (Mediatech, Herndon, VA) supplemented with 2 mmol/L L-glutamine and 10% fetal bovine serum (Hyclone, Logan, UT). The HCT116 colorectal carcinoma cell line and its isogenic derivatives in which the *DNMT1* (1 KO), *DNMT3B* (3B KO), and *DNMT1* and *DNMT3B* (DKO) genes are inactivated were provided by Dr. Bert Vogelstein. Cell lines were treated with 5 μmol/L 5-azadC for 4 days (fresh drug was added every 24 hours) followed by a 24-hour treatment with 100 nmol/L TSA. In some cases, treatment with only one drug at the same concentration and time was also done. All chemicals were purchased from Sigma (St. Louis, MO).

Tumor specimens. Fresh-frozen tumors were obtained from the University of Florida Shands Cancer Center Molecular Tissue Bank and the Department of Neurological Surgery of Asan Medical Center, Seoul, Korea. All specimens and pertinent patient information were treated in accordance with policies of the Institutional Review Board of the University of Florida Health Sciences Center. Tumors were analyzed by a surgical pathologist. Specifics on the number of cases of each type and grade are described in the Results. The tissue samples were divided into two, and one half was pulverized in Trizol (Invitrogen, Carlsbad, CA) for RNA purification according to the manufacturer's instructions, and the other half was used for DNA preparation by the standard proteinase K, phenol/chloroform extraction method (17). Two normal brain DNA and RNA samples from cancer-free individuals were purchased from BioChain (Hayward, CA). Normal tissue RNA samples were also purchased from Clontech (Mountain View, CA).

Expression microarrays. To prepare labeled complementary RNA (cRNA), total RNA was extracted from each sample and prepared for hybridization according to the Affymetrix GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). Briefly, RNA was extracted from cell lines using the RNeasy Mini kit (Qiagen, Valencia, CA). Samples were further cleaned and concentrated with an RNeasy MiniElute Cleanup column (Qiagen). A 200-ng aliquot of each RNA sample was loaded into an RNA 6000 Nano Chip and run on a Bioanalyzer (Agilent Technologies, Palo Alto, CA) to evaluate sample quality. Five micrograms of total RNA were used as a template for cDNA synthesis with the Superscript Choice System kit (Invitrogen). First-strand synthesis was primed with a T7-$(dT)_{24}$ oligonucleotide primer containing a T7 RNA polymerase promoter sequence on the 5′-end (Genset Oligos, St. Louis, MO). Second-strand products were purified with the GeneChip Sample Cleanup Module (Affymetrix) and used as a template for *in vitro* transcription (IVT) with biotin-labeled nucleotides (Bioarray High Yield RNA Transcript Labeling kit, Enzo Diagnostics, Farmingdale, NY). IVT reactions were purified with the GeneChip Sample Cleanup Module, and 20 μg of the product was heated at 94°C for 35 minutes in fragmentation buffer to produce fragments that are 35 to 200 bp in length. Probes were prepared from two independent batches of untreated and treated cells, and each probe mixture was hybridized to a different GeneChip.

For the array hybridization, fragmented samples were submitted to the University of Florida's Interdisciplinary Center for Biotechnology Research Gene Expression Core Facility. A 15-μg aliquot of fragmented cRNA was hybridized for 16 hours at 45°C to an Affymetrix GeneChip U133A array (~14,500 genes). After hybridization, each array was stained with a streptavidin-phycoerythrin conjugate (Molecular Probes, Carlsbad, CA), washed, and visualized with a Genearray Scanner (Agilent Technologies). Images were inspected visually for hybridization artifacts. In addition, quality assessment metrics were generated for each scanned image. Expression values were calculated using Microarray Suite Version 5 software (Affymetrix) to generate *.cel files. Probe Profiler software (v1.3.11; Corimbia, Inc., Berkeley, CA) was used to convert *.cel file intensity data into quantitative estimates of gene expression (EScores). Genes not expressed in any of the samples ($P > 0.05$) were considered absent and were not included in further analyses.

Data analysis. EScores were analyzed using Significance Analysis of Microarrays (SAM; ref. 18). We identified genes considered significantly effected by treatment at both the 1% and 10% false discovery rate (FDR). Genes chosen for further confirmation by reverse transcription-PCR (RT-PCR) were drawn from both the 1% and 10% FDR gene lists. The expression value of those genes considered to be differentially expressed was normalized by performing a Z transformation. Hierarchical clustering was done on the normalized expression values with TreeView.

RT-PCR. RT-PCR was carried out according to standard protocols. Briefly, untreated and treated cells were homogenized in Trizol, and the RNA was purified according to the manufacturer's instructions (Invitrogen). First-strand cDNA synthesis was carried out using Superscript III RT (Invitrogen). Subsequently, the cDNA was used in semiquantitative PCR using the primers listed in Supplementary Table S1. Amplification of β-actin was used as a control for RNA integrity for all samples. Following PCR, reaction products were resolved on 2% agarose gels and photographed using a Bio-Rad gel documentation system.

BGS and MSP. BGS and MSP were done essentially as described previously (19). For a complete listing of primer sequences used for BGS and MSP, refer to Supplementary Table S2. TaqGold (ABI) or Hotmaster Taq (Eppendorf, Westbury, NY) DNA polymerases were used. For BGS, the band was purified from the agarose gel using the QiaexII gel extraction kit (Qiagen) and cloned using the TA Cloning kit (Invitrogen). At least six independent clones from at least two independent PCR reactions were cloned and sequenced using the M13 reverse primer. All sequencing was done at the University of Florida Center for Mammalian Genetics DNA Sequencing Facility. For MSP, reaction optimization was done to ensure that the PCR was in the linear amplification range. Specificity of MSP primers was routinely validated using human sperm genomic DNA unmethylated or *in vitro* methylated with CpG Methylase (New England Biolabs, Ipswich, MA; data not shown). PCR products were resolved on 2% agarose gels.

Plasmid construction. Expression plasmids for CST6, TACSTD2, TAC1, TSPYL5, BIK, and CLIC3 were constructed by first amplifying the complete coding region of each gene from HCT116 DKO cell line cDNA using either

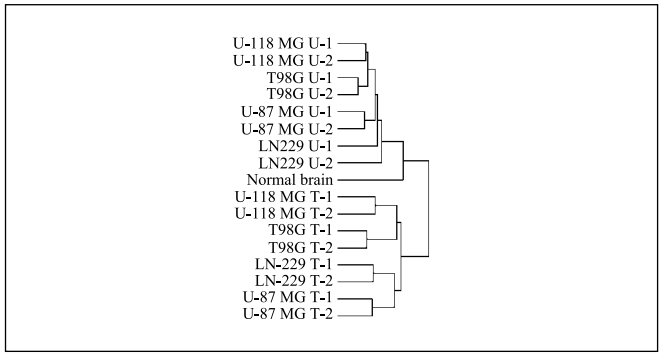

Figure 1. Dendrogram obtained by hierarchical cluster analysis, using all genes up-regulated by SAM analysis with an FDR ≤ 10%, to show the degree of relatedness between each of the cell lines either untreated (*U*) or treated (*T*) with 5-azadC and TSA, and a sample of normal brain from an individual without cancer. Numbers 1 and 2 refer to duplicate samples.

Herculase or *PfuTurbo* DNA polymerases according to the manufacturer's instructions (Stratagene, La Jolla, CA). PCR primers (sequences available upon request) were designed to contain the *Xho*I restriction enzyme recognition site. Following PCR, an aliquot of the reaction was digested with *Xho*I, and the resulting product was cloned into the *Xho*I site of the eukaryotic expression vector pcDNA3.1 (Invitrogen), which also encodes neomycin resistance. Recombinant clones were screened for proper orientation and sequenced. The $p16^{INK4a}$ expression plasmid has been described previously (20).

Colony formation assays. T98G, LN-229, and U-87 MG cells were seeded in six-well plates. The following day, they were transfected using 5 μL of LipofectAMINE and 2 μg of each of the expression plasmids described above according to the manufacturer's instructions (Invitrogen). Forty-eight hours after transfection, the medium was replaced with fresh medium containing G418 to kill untransfected cells. Cells were grown for 9 to 14 days with fresh media and G418 added every 3 days. Once colonies were visible, they were stained with methylene blue and photographed on a Bio-Rad gel documentation system equipped with a white light transilluminator. All assays were repeated at least thrice, and data are presented as the mean ± SD. Separately, an aliquot of RNA was prepared from transfected cells, and the expression of each of the genes was confirmed by RT-PCR (data not shown).

Western blotting. Whole-cell extracts from untreated or drug treated cells were prepared according to standard procedures by directly lysing cells in SDS sample buffer. Proteins were resolved on 16% SDS-PAGE gels, transferred to polyvinylidene difluoride membrane, and Western blotted as described previously (21). Antibodies used for Western blotting include TACSTD2 (ESA, Chemicon, Temecula, CA; 1:500 dilution), CLIC3 (Abcam, Cambridge, MA; 1:500), CST6 (R&D Systems, Minneapolis, MN; 1:500), BIK N-19 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:500), and glyceraldehyde-3-phosphate dehydrogenase (Abcam; 1:2,500).

Table 1. Properties of the 10 genes identified from the microarray screen as epigenetically silenced in glioma, which are characterized in this article

Name	Symbol	Chromosomal location	CpG*	Known or proposed functions	Relationship to cancer/disease
Tumor-associated calcium signal transducer 2	*TACSTD2*	1p32-p31	++	Encodes carcinoma-associated antigen, transduces intracellular calcium signal	Missense mutation L186P in gelatinous drop-like corneal dystrophy
Cytidine deaminase	*CDA*	1p36.2-p35	+	Catalyzes deamination of cytidine and deoxycytidine	Increased expression is associated with resistance to cytosine arabinoside or 5-azadC
Cysteine dioxygenase, type I	*CDO1*	5q22-q23	++	Catabolism of cysteine, an essential precursor for the biosynthesis of glutathione	Cysteine may be limiting in tumors, including gliomas
Ribonuclease T2	*RNASET2*	6q27	++	Novel member of the Rh/T2/S-glycoprotein class of extracellular ribonucleases	Homozygous deletion at 6q27 in ovarian cancer
tachykinin, precursor 1 (substance K, P, neurokinin 1, neurokinin 2)	*TAC1*	7q21-q22	++	Encoding tachykinin peptide hormones that function as neurotransmitters (substance P, neurokinin A, neuropeptide K, and neuropeptide γ)	Mediate cytokine release to alter immune response to tumor, substance P may promote astrocyte growth under certain conditions
KIAA1750 protein	*TSPYL5*	8q22.1	++	Homology to testis specific Y-encoded (TSPY), belongs to NAP family	Frameshift mutation at codon 153 in SIDDT for TSPYL1
Chloride intracellular channel 3	*CLIC3*	9q34.3	++	Stimulates chloride ion channel activity, interacts with ERK7	Unknown
Cystatin E/M	*CST6*	11q13	++	Potent inhibitor of lysosomal cysteine proteases, suppresses cell proliferation, migration, and invasion	Down-regulated in metastatic breast cancer
Bcl-2 interacting killer	*BIK*	22q13.31	++	Proapoptotic gene in the Bcl2 family, shares critical BH3 domain with other death-promoting proteins, Bax and Bak	Mutated in B-cell lymphoma, deleted in ~30% of human gliomas
E2F-like protein	*TFDP3*	xq26.2	++	Component of the E2F/DP transcription factor complex	Unknown

*++, CpG island; +, CpG sites (non-island).

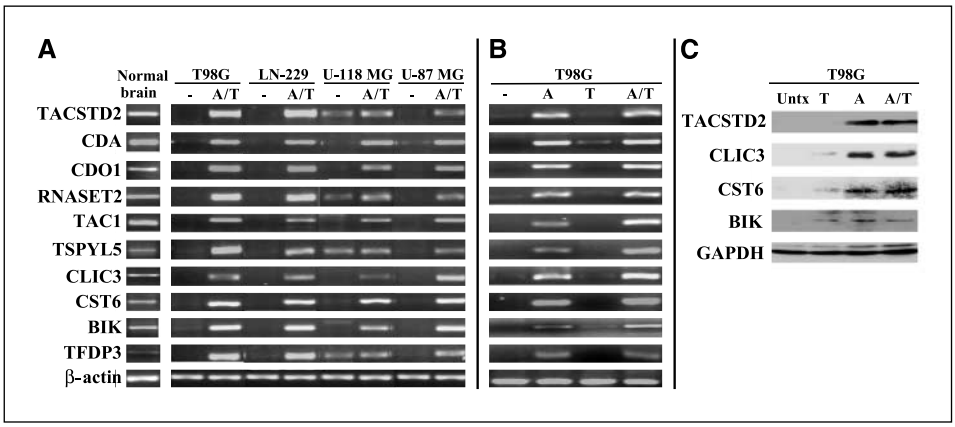

Figure 2. *A,* RT-PCR confirmation of gene expression changes identified in the microarray analysis for a panel of 10 genes of interest. RNA was prepared from untreated and treated cells (5 μmol/L 5-azadC for 96 hours plus 100 nmol/L TSA for a final 24 hours; *A/T*) and used in semiquantitative RT-PCR. Expression analysis from a representative sample of normal brain. Similar results were obtained with an additional two samples of normal brain (data not shown). *B,* effects of individual drug treatments on the expression of 10 genes monitored by RT-PCR. T98G cells were untreated (−) or treated with 5 μmol/L 5-azadC for 96 hours (*A*), 100 nmol/L TSA for 24 hours (*T*), or 5-azadC plus TSA (*A/T*). β-Actin is used as a loading control for all gene expression analyses. *C,* Western blotting analysis of select genes from (*A*). Treatments are the same as in (*B*); *left,* antibody used. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) antibody served as a loading control.

Results

Identification of genes induced by 5-azadC and TSA in glioma cell lines. To identify novel aberrantly hypermethylated genes in glioma cells, we treated four glioma cell lines (T98G, LN-229, U-118 MG, and U-87 MG) with a combination of 5-azadC and TSA. This was done in an effort to maximize reactivation of as many epigenetically silenced genes as possible because these agents are known to act synergistically (9). Specifically, cells were treated for 4 days with 5 μmol/L 5-azadC alone (added fresh to the media each day) followed by a 24-hour treatment with 100 nmol/L TSA. Global changes in gene expression were determined using the Affymetrix U133A GeneChip, which allows for the analysis of ~14,500 transcripts. Once the analysis was completed, the data were analyzed by SAM software, and we narrowed down the number of potential targets by selecting only those genes whose expression was changed ≥2-fold in at least three of four cell lines from two independent RNA preparations. Hierarchical clustering of the four cell lines, treated and untreated, and one sample of normal brain from an individual without cancer is shown in Fig. 1. The dendrogram shows that the two independent biological replicates for each cell line and drug treatment clustered together (were the most similar), indicating that our methods are reproducible. A total of 160 genes meeting our criteria were up-regulated, and 14 genes were down-regulated following 5-azadC and TSA treatment. A complete list of these genes is given in Supplementary Table S3. We identified genes, such as *stratifin*, which have been found to be silenced by aberrant promoter hypermethylation in other tumor types (22), and MAGE genes (*MAGEA9* and *MAGEB2*), which are normally methylated in cells and have been identified in other microarray screens using DNA methylation inhibitors (23), thus validating our screening procedures. We further narrowed the list of genes for follow-up confirmation and functional study by selecting primarily those up-regulated genes that contain a CpG island within their 5′-regulatory region (with one exception). Supplementary Table S4 lists the characteristics of the 38 genes that met our criteria. Genes listed in Supplementary Tables S3 to S4 include regulators of cell growth, transcription, cell-matrix interactions, brain function, and others where the function is unknown.

Expression analysis of select genes in glioma cell lines and the effects of individual drug treatments. For the 38 genes listed in Supplementary Table S4, primers were designed to confirm the microarray expression data using semiquantitative RT-PCR on the same four glioma cell lines used in the initial screen and two others (LN-18 and DBTRG-05MG). We were able to show that the majority of the 38 genes were markedly up-regulated in at least three of the six glioma cell lines, thus validating our microarray methods. RT-PCR expression data on the 38 genes in six glioma cell lines, including the degree of basal expression, the change in expression following 5-azadC and TSA treatment, and the expression in normal brain, is summarized in Supplementary Table S4. We further narrowed our focus to the 10 genes shown in Table 1 and Fig. 2. These genes include *tumor-associated calcium signal transducer 2* (*TACSTD2*), *cytidine deaminase* (*CDA*), *cysteine dioxygenase, type I* (*CDO1*), ribonuclease T2 (*RNASET2*), *tachykinin precursor 1* (*TAC1*), *TSPY-like 5* (*TSPYL5*), *chloride intracellular channel 3* (*CLIC3*), *cystatin E/M* (*CST6*), *Bcl-2 interacting killer* (*BIK*), and *transcription factor DP family, member 3* (*TFDP3*). Properties of the 10 genes are summarized in Table 1. All genes contain a CpG island within their 5′-flanking region (with the exception of *CDA*), show very low or undetectable expression in most glioma cell lines, are robustly up-regulated following 5-azadC plus TSA treatment, and are expressed in normal brain tissue (Fig. 2*A*). With the exception of BIK, PubMed literature searches indicated that none of the genes have been reported to be silenced in tumors by aberrant DNA methylation. BIK was reported to be down-regulated by histone deacetylation in a lung cancer cell line (24) and up-regulated by 5-azadC treatment in a renal cell cancer cell line (25), although the DNA methylation status of BIK was not examined in the latter study.

Because cells were treated with both 5-azadC and TSA in the initial microarray screen, it remained possible that a subset of the up-regulated genes are not methylated but rather are controlled by histone deacetylation, as has been reported for the $p21^{WAF1/CIP1}$ gene (8). To test this possibility, we treated T98G cells with 100 nmol/L TSA alone for 24 hours and 5 μmol/L 5-azadC alone for 96 hours then analyzed the expression of the same 10 genes by RT-PCR (Fig. 2*B*). Results indicate that expression of TACSTD2,

CDO1, RNASET2, TAC1, TSPYL5, CLIC3, CST6, and TFDP3 was not affected by TSA alone, but that their expression was robustly induced by 5-azadC. Interestingly, CDA and BIK were weakly induced by TSA but more strongly by 5-azadC, suggesting that their regulation is influenced to a greater degree by histone modifications than the other eight genes in the panel. Similar results were also obtained using the LN-229 and U-87 MG cell lines (data not shown). RT-PCR results were confirmed by Western blotting for four genes (*TACSTD2, CLIC3, CST6*, and *BIK*) where an antibody was commercially available (Fig. 2*C*). Data summarizing the expression patterns of all 10 genes are shown in Table 2. Taken together, our results clearly show that 5-azadC treatment of T98G cells yields the highest level of gene reexpression, suggesting that DNA methylation is the dominant silencing mechanism.

DNA methylation analysis of epigenetically regulated genes in glioma cell lines. Strong induction of expression in the presence of a DNA methylation inhibitor suggests that a gene is a target of aberrant promoter DNA methylation; however, it remains possible that the induction is an indirect effect. We therefore designed MSP primers within the CpG island regions for each of the 10 genes (Fig. 3, *left*) to analyze their DNA methylation status. MSP is a semiquantitative method for determining methylation status of CpG sites within a primer binding site using sodium bisulfite-modified genomic DNA as a template (26). MSP results from the cell lines and normal brain tissue allowed for the 10 genes to be divided into three classes (Fig. 3, *right*). Class I genes are hypomethylated in normal brain and either partially or fully methylated in the majority of the tumor cell lines (*TAC1, TSPYL5, CST6, BIK*, and *CDO1*). Class II genes show roughly equal amounts of unmethylated and methylated alleles by MSP in the normal brain sample. The majority of the tumor cell lines, however, show partial or complete loss of the unmethylated fraction (*TACSTD2* and *CLIC3*). Finally, class III genes are predominantly or fully hypermethylated in normal brain and the glioma cell lines (*CDA, RNASET2*, and *TFDP3*). All MSP results are summarized in Table 2. Up-regulation of *CDA* by 5-azadC may be because of its involvement in the catabolism of 5-azadC itself and could therefore represent a cell survival or stress response (27).

To analyze DNA methylation patterns in greater detail, we did BGS on all 10 genes in the T98G cell line and a sample of normal brain. BGS, like MSP, makes use of sodium bisulfite–modified genomic DNA as a template for PCR; however, for BGS, the PCR primers do not contain CpG sites so that they amplify the template in a manner unbiased by DNA methylation status (28). PCR products are then cloned and sequenced, and the methylation status of all CpG sites between the two primers was determined. The BGS data for the class I and II genes (*TAC1, CST6, BIK, CDO1, TACSTD2*, and *CLIC3*; *TSPYL5* will be discussed later) is shown in Fig. 4, and the total percent methylation across all CpG sites from at least six independent clones is summarized in Table 2. The MSP and BGS data for the class I and II genes are consistent and show that the regions analyzed are hypomethylated in the normal brain and hypermethylated in T98G cells (with the exception of *CDO1*, which, in contrast to the other genes, was only sparsely methylated in T98G). Class I and II genes were also analyzed for DNA methylation by BGS in LN-229 cells (Supplementary Fig. S1), and results were generally similar to T98G. Interestingly, *CDO1* was more heavily methylated, and *BIK* was less methylated in LN-229 cells, consistent with the MSP data. Class III genes were also similarly analyzed (Supplementary Fig. S2; Table 2). CDA was more densely methylated in T98G cells than normal brain, although it does not contain a CpG island. TFDP3, in contrast, was hypermethylated in both the cell line and normal brain, suggesting that methylation is part of its normal biology.

Table 2. Summary of all gene expression and DNA methylation (MSP and BGS) results in brain tumor cell lines and primary tumors

	Gene expression*			Promoter methylation				
				MSP†		BGS‡ (%)		
Gene symbol	Normal brain	BT cell lines (6)	BT tissues (17)	Normal brain	BT cell lines (6)	Normal brain	T98G cell line	BT tumor tissue (MSP)
TAC1	++	−6	−10 (59%)	**U**/M	M (6)	5	93	40
TSPYL5	++	−5, ++ 1	−14 (82%)	**U**/M	M (6)	3	98	93
CST6	++	−6	−15 (88%)	**U**/M	M (6)	6	95	57
BIK	++	−4, +2	−10 (59%)	**U**/M	M (4)	2	40	30
CDO1	++	−5, ++1	NT	**U**/M	**U**/M (4)	0	18	NT
TACSTD2	++	−5, +1	−17 (100%)	U/M	M (6)	32	94	63
CLIC3	++	−6	−10 (59%)	U/M	M (5)	27	100	43
CDA	++	−6	NT	U/M	U/M (5)	23	80	NT
RNASET2	++	−5, +1	NT	U/**M**	M (5)	2	5	NT
TFDP3	+	−4, +2	NT	M	M (5)	90	83	NT

Abbreviations: U, unmethylated; M, methylated; NT, not tested.

*++: moderate, +: weak, −: no expression by RT-PCR out of the total number of cell lines/samples indicated in parentheses.

†Bold type indicates dominant signal when a mixture of U and M are present. The number of cell lines with the indicated methylation pattern is indicated in parentheses.

‡Number denotes the % methylation across all CpG sites and all clones.

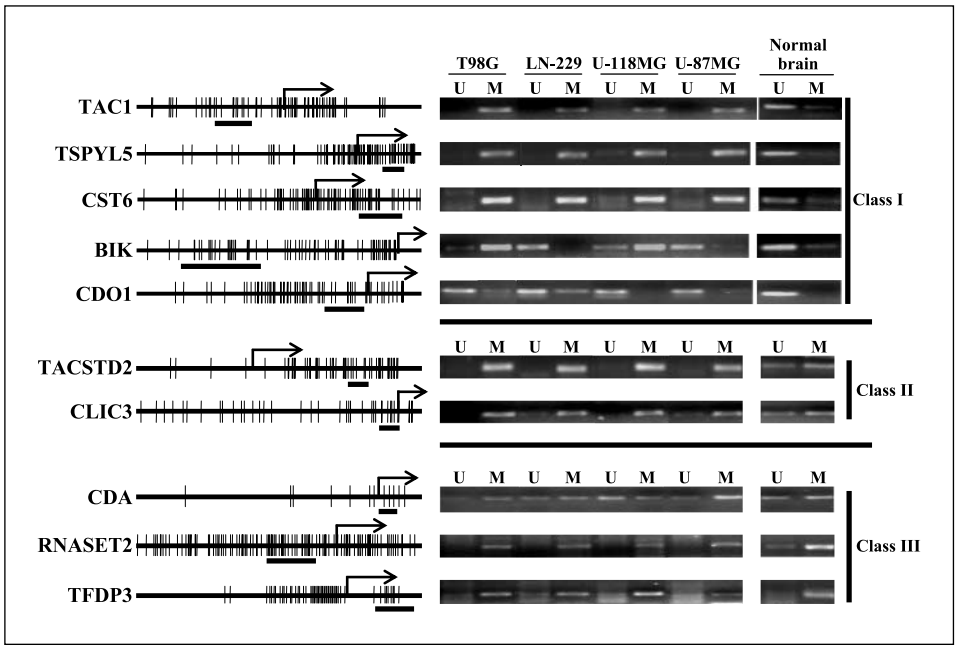

Figure 3. MSP DNA methylation analysis of 10 genes identified in the microarray screen as being up-regulated by 5-azadC and TSA treatment. CpG plots of the 5′-regulatory regions of each of the genes (CpGs, *vertical tick marks*), along with the location of the region analyzed by MSP (*thick horizontal line* below CpG plot). *U,* PCR reaction with unmethylated DNA-specific primers; *M,* PCR reaction with methylated DNA-specific primers. All reactions were done at least three times and were in the linear amplification range. Normal brain DNA is from an individual without cancer. Results were similar with two additional normal brain samples (data not shown). Division of genes into classes I, II, and III is discussed in the text.

Curiously, RNASET2 was hypomethylated in both T98G cells and normal brain, inconsistent with the MSP data. As we were unable to analyze the MSP region using BGS, due to difficulties in obtaining BGS PCR primers in this region, we cannot rule out the possibility of localized methylation within the MSP region or that RNASET2 is indirectly regulated by epigenetic mechanisms. In any case, class I genes represent the best candidates for putative growth-regulatory genes and biomarkers because their methylation

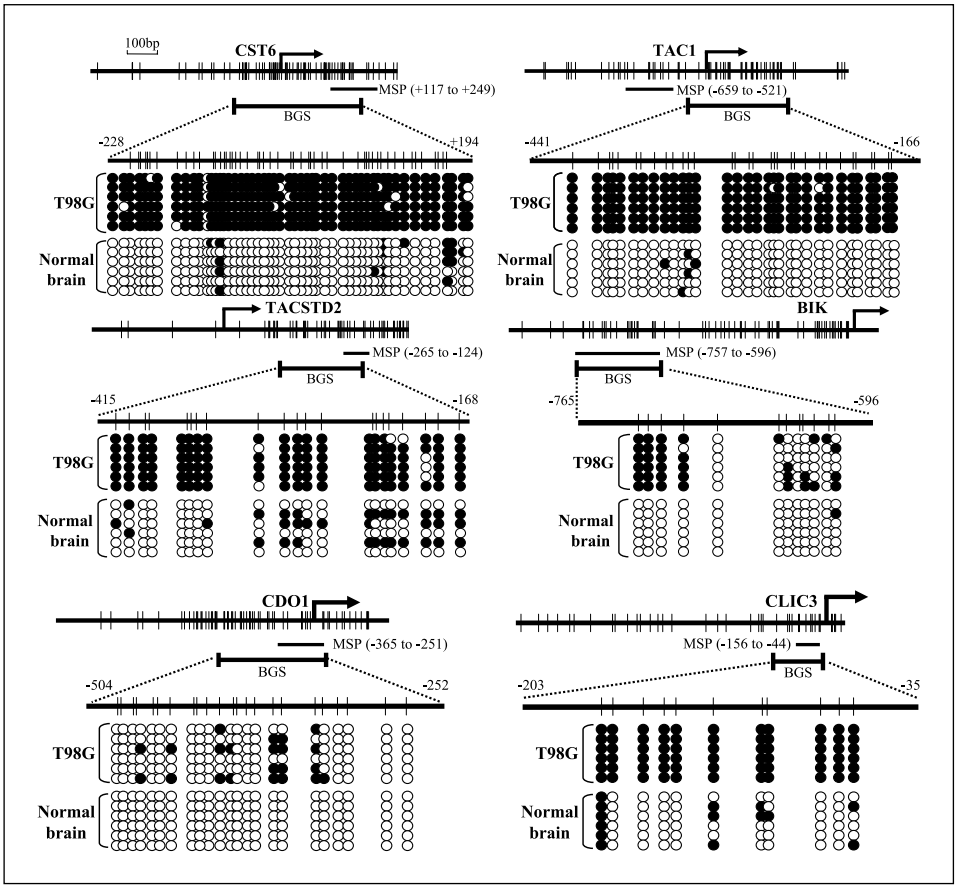

Figure 4. BGS analysis for six of the genes in Fig. 3 (*CST6, TAC1, TACSTD2, BIK, CDO1*, and *CLIC3*) in the T98G cell line and normal brain. The CpG plot for each gene (*top*) indicates the location of the BGS region relative to the region analyzed by MSP. A blow-up of the region analyzed by BGS (*bottom*) with tick marks denoting the position of CpG dinucleotides. BGS results are summarized with filled circles representing methylated CpGs or open circles representing unmethylated CpGs. Each line is an independently sequenced clone. The bent arrow indicates the position of the transcription start site as defined using NCBI Map Viewer (http://www.ncbi.nlm.nih.gov/mapview). Numbering is relative to the transcription start site for BIK and relative to the ATG start codon for CST6, TAC1, TACSTD2, CDO1, and CLIC3.

is specific to the transformed state. The presence of partially methylated TACSTD2 and CLIC3 clones from normal brain suggests that cells may have a heterogeneous methylation pattern or that certain cell types are hypermethylated, whereas others are hypomethylated. The BIK promoter region is clearly more heavily methylated in T98G cells relative to normal brain; however, the methylation is not as dense as in other promoters we examined (compare with CST6, TAC1, and TACSTD2 in Fig. 4) and may, in part, explain why BIK expression is reactivated by TSA treatment alone, whereas the more heavily methylated genes are not (Fig. 2*B*). Taken together, the MSP and BGS results indicate that the genes we identified in the initial microarray screen, particularly the class I genes, are bona fide DNA methylation targets in glioma cell lines, that our MSP and BGS results are in good agreement, and that hypermethylation of the TAC1, TSPYL5, CST6, BIK, CDO1, and CLIC3 promoter regions is not part of their normal biology in brain tissue.

Expression and DNA methylation analysis of class I and II genes in primary brain tumor tissue. Class I genes (*CST6, TAC1, BIK*, and *TSPYL5*) and class II genes (*TACSTD2* and *CLIC3*) showed a high frequency of DNA methylation-mediated silencing in glioma cell lines (Figs. 3 and 4). Because *CDOI* (a class I gene) was only sparsely methylated in T98G cells, we did not include it in further analyses. We next sought to extend the analysis of expression and DNA methylation status of these six genes to a panel of snap-frozen primary brain tumor samples. We obtained 30 brain tumors, including 19 glioblastoma multiforme (grade 4), 2 high-grade gliomas, 7 astrocytomas (grade 3), and 2 oligodendrogliomas. Genomic DNA for MSP DNA methylation analysis was isolated from all 30 tumors. Matched normal was not available because adjacent normal tissue is not routinely removed during brain tumor surgery. Figure 5*A* shows a representative subset of the MSP data we obtained from four primary tumors and one normal brain sample. Results indicate that the frequency of hypermethylation in the primary tumors varies among the six genes. Most significantly, methylation of the *TSPYL5* promoter region was detectable in nearly 100% of the primary tumors across all grades and types, indicating that this may be an early event (Table 3). Methylated alleles of TAC1, CST6, BIK, TACSTD2, and CLIC3 were detected in 40%, 57%, 30%, and 63%, and 43%, respectively, of all tumor samples by MSP, indicating that hypermethylation of class I and II genes is frequent in both glioma cell lines and primary glial tumors (Table 3). We also observed amplification with the unmethylated DNA-specific MSP primers in several of the tumor samples (e.g., see TAC1 T9 and T10 and BIK, T35 and T37; Fig. 5*A*), and this is likely due to normal cells present within the surgically removed tumor sample. This is not surprising given that there is no clear margin or capsule between the tumor and the normal tissue and because of the invasive nature of glioma. Interestingly, the frequency of methylation of CST6, TACSTD2, and TAC1 was higher in the more malignant, higher-grade tumors, although the number of tumors analyzed was not sufficiently large to make statistically significant assessments. BIK was methylated in 30% of the tumors overall, and this is likely a significant underestimate of its epigenetic silencing frequency because it may be down-regulated by histone deacetylation alone (Fig. 2*B* and Fig. 4; ref. 24). This notion is supported by the expression analysis presented in the next section, where it is down-regulated at nearly twice the frequency of methylation.

Sufficient quantities of RNA for analysis were obtained from 17 of the 30 tumor samples. We did semiquantitative RT-PCR for the class I and II genes in these 17 tumor samples and compared this with a sample of normal brain (Fig. 5*B*). The majority of tumors showed reduced transcript levels of all six genes relative to normal brain. We confirmed these results with an additional two samples of normal brain (data not shown). Transcript levels of TAC1, CST6, BIK, TACSTD2, and CLIC3 were reduced at a frequency equal to or greater than the frequency of methylation determined by MSP. Reduced TSPYL5 expression was slightly less frequent than TSPYL5 promoter hypermethylation and is probably due to differences in the sensitivity of MSP and RT-PCR. It also suggests that some of the genes, such as *BIK*, are down-regulated by other epigenetic mechanisms, such as histone deacetylation. Taken together, the analysis of DNA methylation and expression of the class I and II genes reveals that promoter hypermethylation and transcriptional down-regulation is common in primary brain tumors, similar to the results obtained with glioma cell lines. A complete summary of all expression and DNA methylation data (MSP and BGS) for both cell lines and primary tumors is shown in Table 2. Hypermethylation and down-regulation of the *TSPYL5, CST6*, and

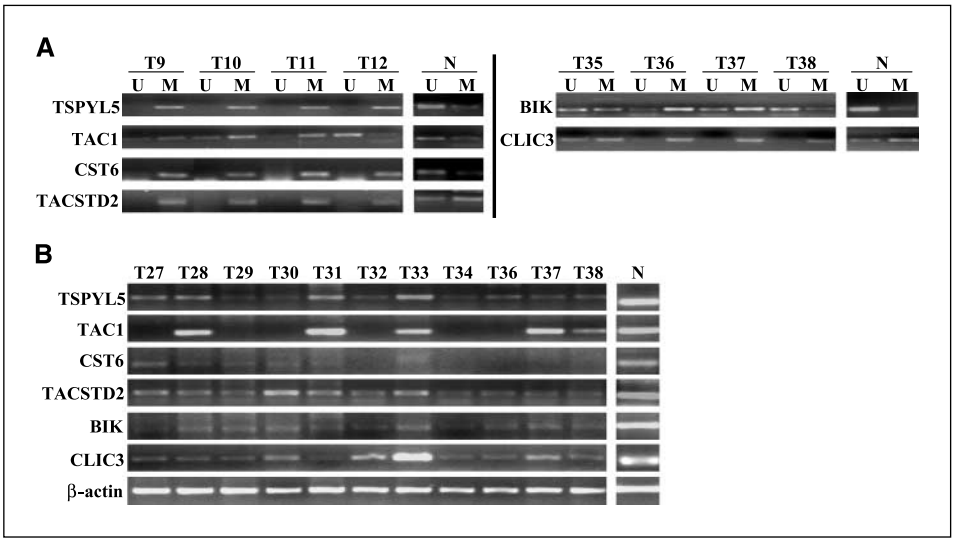

Figure 5. Analysis of DNA methylation and expression of six class I and II genes in snap-frozen primary brain tumors. *A,* representative MSP results from four tumor samples (out of thirty, denoted above the gels by *T#*) and one normal brain sample (*N*) for TSPYL5, TAC1, CST6, TACSTD2, BIK, and CLIC3. Abbreviations are the same as in Fig. 3. *B,* semiquantitative RT-PCR expression analysis of the same six genes in eleven tumor samples. One representative normal brain sample is shown at the far right. Not all tumor samples could be evaluated for expression due to limited quantities of tumor material.

Table 3. Summary of gene expression and DNA methylation (MSP) data, according to pathologic classification, for primary brain tumor samples

	Tumor type, *n* (%)				
	Glioblastoma multiforme	High-grade glioma	Astrocytoma	Oligodendroglioma	Total for all tumors
Methylation frequency*					
No. tumors analyzed	19	2	7	2	30
TAC1	8 (42)	1 (50)	1 (14)	2 (100)	12 (40)
TSPYL5	19 (100)	2 (100)	5 (71)	2 (100)	28 (93)
CST6	14 (74)	1 (50)	1 (14)	1 (50)	17 (57)
BIK	5 (26)	0 (0)	2 (28)	2 (100)	9 (30)
TACSTD2	13 (68)	0 (0)	4 (57)	2 (100)	19 (63)
CLIC3	6 (32)	1 (50)	4 (57)	2 (100)	13 (43)
Gene down-regulation frequency†					
No. tumors analyzed	9	1	6	1	17
TAC1	5 (59)	1 (100)	4 (67)	0 (0)	10 (59)
TSPYL5	8 (89)	1 (100)	4 (67)	1 (100)	14 (82)
CST6	7 (78)	1 (100)	6 (100)	1 (100)	15 (88)
BIK	6 (67)	1 (100)	3 (50)	0 (0)	10 (59)
TACSTD2	9 (100)	1 (100)	6 (100)	1 (100)	17 (100)
CLIC3	6 (67)	0 (0)	3 (50)	1 (100)	10 (59)

*Number of tumors analyzed showing hypermethylated alleles out of the total number analyzed.
†Expression is down-regulated relative to the normal brain by RT-PCR.

TACSTD2 genes was particularly noteworthy and suggests an important function for these genes in the development or progression of gliomas.

Re-expression of class I and II DNA methylation target genes suppresses the growth of glioma cells in culture. Although growth suppressive effects have been established for CST6 in breast cancer (29), the effects of TSPYL5, CST6, TACSTD2, and CLIC3 expression on glioma cell growth has not been investigated. It was suggested that one of the TAC1-encoded peptides, substance P, enhances proliferation in a glioma xenograft model (30), and BIK induces apoptosis in glioma cells when reexpressed using a viral vector (31). To ascertain whether these six genes have growth suppressive properties in glioma cell lines in which the endogenous gene is methylated, we first cloned their full-length cDNAs into the pcDNA3.1 mammalian expression vector (also encoding neomycin resistance). G418 is added at a dose predetermined to kill 100% of the untransfected cells within 9 to 14 days. Expression plasmids were transfected into the T98G, LN-229, and U-87 MG cell lines. Empty parental vector was used as a negative control, and the number of colonies forming in this reaction was set at 100%. Transfection of the known tumor suppressor gene, $p16^{INK4a}$, was used as a positive control and resulted in marked suppression of growth in all cell lines (Fig. 6*A* and *B*). Results varied slightly among the three lines; however, reexpression of TSPYL5 and BIK resulted in the most pronounced suppression of growth across all cell lines (Fig. 6). TACSTD2 was a potent suppressor of growth in LN-229 and U-87 MG cells. This may be due to differences in the complement of other epigenetic and genetic changes in these cells. CST6 and TAC1 expression had modest effects on cell growth. Given that CST6 is believed to regulate cell adhesion and metastasis, this result may not be unexpected. CLIC3 had no affect on cell growth in this assay. We confirmed expression of each of the transfected genes by RT-PCR (data not shown). Results using the colony formation assay, therefore, show that TSPYL5, BIK, and TACSTD2 expression yield a pronounced and reproducible growth suppressive effect followed by CST6, TAC1, and then CLIC3. Taken together, these results suggest that TSPYL5, BIK, and TACSTD2 have tumor-suppressor properties in glioma cells.

Differential expression of TSPYL family members in normal human tissues and evidence that other TSPYL genes are targeted for epigenetic silencing in brain tumors. Our results indicated that *TSPYL5* was methylated in nearly 100% of primary tumors (Figs. 2-3; Tables 2-3). Furthermore, TSPYL5 suppressed glioma cell growth as well and the known tumor-suppressor gene $p16^{INK4a}$. Interestingly, TSPYL5 is a member of gene family that includes TSPYL1, TSPYL2, TSPYL3, TSPYL4, and TSPYL6 (32). In Fig. 7, we analyzed the DNA methylation status of the TSPYL5 promoter region in greater detail by BGS. The 5′-regulatory region of TSPYL5 is heavily methylated in T98G and LN-229 cells and almost completely devoid of methylation in normal brain tissue (Fig. 7*A*; Supplementary Fig. S1). We further analyzed TSPYL5 methylation by performing BGS on three primary glioblastoma samples. All tumors showed high levels of methylation (Supplementary Fig. S3) consistent with down-regulation of TSPYL5 expression in these samples (Fig. 5; data not shown). We then examined the expression of TSPYL1, TSPYL2, TSPYL3, TSPYL4, and TSPYL6 in untreated T98G cells and found that TSPYL1 and TSPYL4 were expressed, whereas TSPYL2, TSPYL3, and TSPYL6 were transcriptionally silenced but induced by 5-azadC alone or 5-azadC plus TSA treatment (Fig. 7*B*). We also examined the expression of the TSPYL genes in a subset of primary brain tumors. Similar to T98G cells, TSPYL1 and TSPYL4 expression was readily detected at levels comparable with normal brain. TSPYL2, TSPYL3, and to a lesser extent TSPYL6, however, were down-regulated in

most of the tumor samples (Fig. 7*C*). Finally, we analyzed expression of the TSPYL genes in a panel of normal human tissues. Interestingly, all TSPYL genes were highly expressed in both whole adult and fetal brain. The TSPYL genes were variably expressed in other tissues, with expression of TSPYL*4* and TSPYL5 being more ubiquitous and TSPYL1, TSPYL2, TSPYL3, and TSPYL6 being more restricted (Fig. 7*D*). Collectively, these results reveal that the TSPYL family of genes is highly expressed in normal brain, and that TSPYL2, TSPYL3, and TSPYL6 may also be subject to frequent epigenetic silencing in brain tumors.

Role of individual DNMTs in mediating promoter hypermethylation of select class I and II genes. Increasing evidence suggests that DNMT1, DNMT3A, and DNMT3B work cooperatively to methylate some regions of the genome, whereas methylation of other regions is mediated primarily by one of the DNMTs (33). To determine which of the DNMTs may mediate aberrant hypermethylation of class I and II genes, we employed a model cell line system (i.e., the HCT116 colon cancer cell line and its isogenic derivatives in which the *DNMT1, DNMT3B*, and *DNMT1* and *DNMT3B* genes have been genetically disrupted; KO; ref. 34). We found that untreated parental HCT116 cells, like many of the glioma cell lines and primary tumors, show reduced or absent expression of TACSTD2, TAC1, TSPYL5, and BIK (Fig. 8*A*). CST6 and CLIC3 were not informative because they were expressed equally in the parental and DNMT knockout cells (data not shown). In keeping with the expression results, MSP shows that parental HCT116 cells contain predominantly hypermethylated TACSTD2, TAC1, TSPYL5, and BIK promoters (Fig. 8*B*), supporting our view that these cells are, in general, a valid model for studying methylation of these four genes in malignant glioma. TAC1 and TSPYL5 were reexpressed and demethylated in HCT116 cells only if both the *DNMT1* and *DNMT3B* genes were inactivated (Fig. 8*A* and *B*), suggesting that both DNMTs cooperate to methylate these promoter regions. In contrast, BIK expression was up-regulated in DNMT3B and DKO cells, indicating that DNMT3B may be particularly important in methylating this region. TACSTD2 expression was maximal in DKO cells where the promoter was hypomethylated (Fig. 8). Taken together, these results show that the HCT116 colon cancer cell line system is a valuable model for studying the role of individual DNMTs in mediating aberrant promoter hypermethylation events and suggests that the genes in our panel are differentially targeted by DNMT1 and DNMT3B.

Discussion

In the present article, we have used a combined approach of pharmacologic inhibition of epigenetic modifications (DNA methylation and histone deacetylation) and gene expression microarrays, to identify genes subject to epigenetic silencing in glioma cell lines. This strategy has proven to be a powerful approach for identifying targets of epigenetic silencing in other tumor types (35, 36). Gliomas are the most common form of central nervous system tumor in adults, and current treatments result in a very poor prognosis, with most patients succumbing to the disease with 1 year of diagnosis. Therefore, it is imperative to learn more about the etiology of gliomas and to develop new disease biomarkers. Our approach identified a panel of 160 genes up-regulated with high frequency in glioma cell lines. We confirmed the microarray data for 38 of these genes and further characterized 10 of them. Using MSP and BGS, we showed that these 10 genes were methylated in glioma cell lines. We also showed that *TAC1, TSPYL5, CST6, BIK, TACSTD2*, and *CLIC3* were methylated to varying but significant degrees in a panel of 30 primary brain tumor samples. Results with TSPYL5 were particularly noteworthy because this gene was methylated in nearly 100% of the tumors. Growth suppression assays showed that TSPYL5, BIK, TACSTD2, and to a lesser extent CST6 and TAC1, exhibited growth suppressive properties, consistent with them having tumor or metastasis suppressor functions in brain tumors. Lastly, we examined the expression of the entire known TSPYL family of genes, and our data indicate that TSPYL2, TSPYL3, and TSPYL6, in addition to TSPYL5, may also be

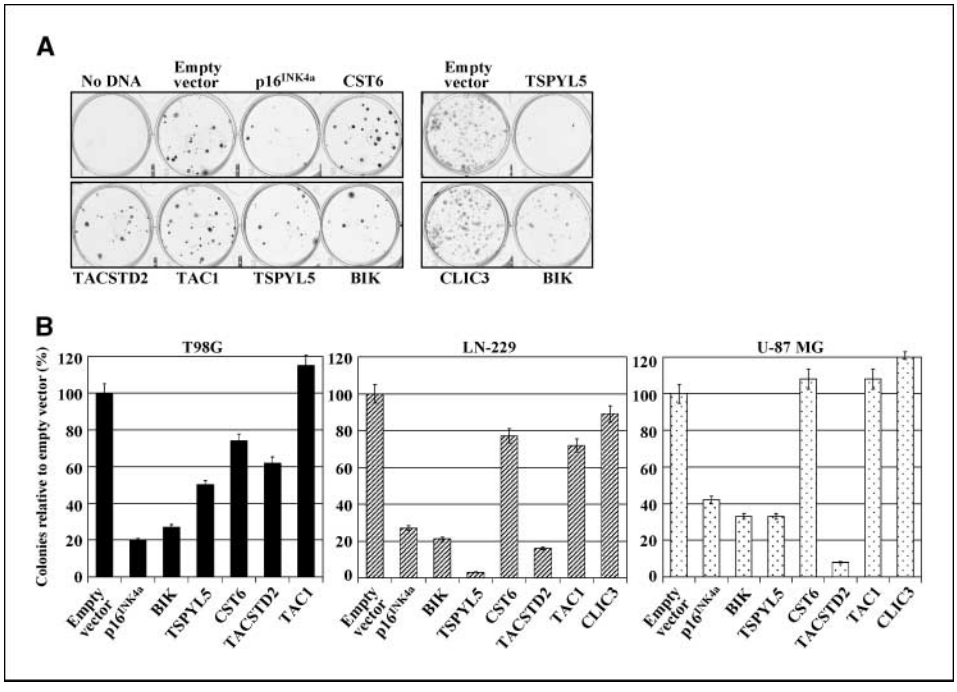

Figure 6. Growth suppression assay. *A,* representative colony formation assay results in T98G (*left*) and LN-229 (*right*) cells following transfection of each of the indicated plasmids and selection in media containing 800 μg/mL G418 for 9 to 14 days. Colonies were then stained with methylene blue and photographed. *B,* quantification of colony formation results from three glioma cell lines: T98G, LN-229, and U-87 MG. Average number of colonies in each transfection relative to the pcDNA3.1 empty vector control (set at 100%). All assays were done at least three times. *Columns,* mean; *bars,* SD. Transfection of *p16*^{INK4a} is used as a positive control for growth suppression because it is a known tumor suppressor gene. CLIC3 was not analyzed in T98G cells.

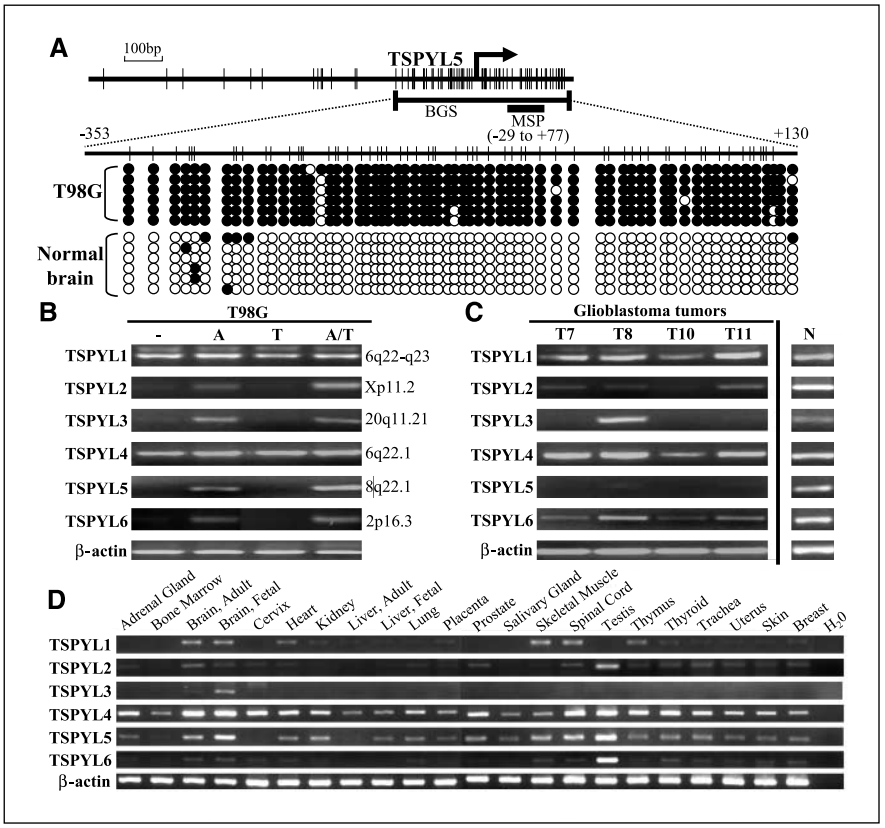

Figure 7. The TSPYL gene family in brain tumors and normal tissues. *A,* BGS analysis of TSPYL5 in T98G cells and normal brain. Symbols are the same as in Fig. 3, and numbering is relative to the ATG start codon. *B,* RT-PCR expression analysis of all TSPYL genes in T98G cells untreated (−), treated with 5 μmol/L 5-azadC (*A*), 100 nmol/L TSA (*T*), or both (*A/T*) under the conditions described in Fig. 2*B*. *C,* RT-PCR expression of TSPYL genes in four primary glioblastoma multiforme samples and one representative normal brain tissue sample. *D,* expression of TSPYL1 to TSPYL6 in a panel of 22 normal human tissues.

frequently targeted for epigenetic silencing in brain tumors, and that all of the TSPYL genes are highly expressed in normal brain. This study, therefore, provides the basis for future work aimed at characterizing the role of these genes in gliomagenesis and provides a wealth of other potential gene targets for future characterization.

Based on MSP data, we divided the 10 genes into three classes. Class I genes in particular, but also class II genes, represent the best candidates for disease biomarkers and/or novel growth regulatory genes in glioma. Class I genes were hypomethylated in normal brain, whereas class II genes showed a mixture of methylated and unmethylated alleles in normal brain. *CDO1*, a class I gene involved in regulating intracellular cysteine levels, a process that has recently been suggested to play an important role in glioma cells by influencing glutathione production and therefore levels of reactive oxygen species (37), was sparsely methylated in T98G but heavily methylated in LN-229 cells. A more comprehensive analysis of CDO1 may, therefore, be warranted in future studies. *CLIC3*, also a class II gene frequently methylated in cell lines and primary tumors, did not display any growth suppressive effects. CLIC3 function is largely unknown, although it has been shown to interact with the MAPK extracellular signal-regulated kinase 7, suggesting that may possess growth-regulatory functions (38). RNASET2 was predominantly methylated in normal brain by MSP, although it did show loss of the minor unmethylated fraction in tumor cell lines, as is seen for the class II genes. Given that it is located in a region of 6q27 commonly deleted in ovarian cancer, and it suppresses the metastatic potential of an ovarian cancer cell line, further study of this gene in glioma may also be justified (39).

Our data revealed for the first time that cystatin E/M (CST6) was frequently targeted for aberrant promoter methylation in glioma cell lines and primary brain tumors. CST6 methylation was detected in 74% of glioblastoma multiforme/high-grade glioma samples and was more frequent in higher-grade than lower-grade tumors. CST6 is a member of the cystatin family. Cystatins are naturally occurring inhibitors of lysosomal/endosomal cysteine

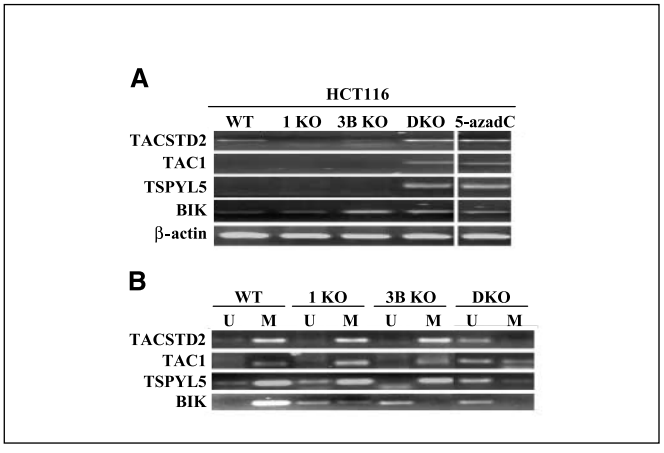

Figure 8. Role of individual DNMTs in methylating class I and II genes. *A,* RT-PCR expression analysis for four informative class I/II genes (those showing reduced or absent expression in parental HCT116 cells), *TACSTD2, TAC1, TSPYL5*, and *BIK*, in parental HCT116 cells (*wt*) and HCT116 cells in which the *DNMT1* (*1KO*), *DNMT3B* (*3BKO*), or both *DNMT1* and *DNMT3B* (*DKO*) genes have been disrupted. Treatment of HCT116 cells with 5 μmol/L 5-azadC for 72 hours is also shown. *B,* MSP DNA methylation analysis on the same genes and in the same cell lines as in (*A*).

proteases (such as papain and certain cathepsins) and act by forming high-affinity reversible complexes with their target proteases (40). Loss of the intricate balance between proteases and protease inhibitors is thought to be essential for the ability of tumor cells at the primary site to detach, become motile, penetrate connective tissues and basement membranes, and establish residence at a distant site (41). This process could be mediated by up-regulation of proteases and/or down-regulation of protease inhibitors like CST6. Indeed, CST6 is down-regulated in metastatic breast tumors and suppresses the growth and invasiveness of breast cancer cells, making CST6 a bona fide metastasis suppressor gene (29). Consistent with silencing of a protease inhibitor, one of the hallmarks of gliomas is their marked ability to invade normal brain tissue. This particularly sinister property of gliomas makes cure by surgery alone essentially impossible. Future studies will be aimed at more directly assessing the effect of CST6 expression on glioma cell motility.

TACSTD2 (TROP-2), or tumor-associated calcium signal transducer-2, is a cell surface glycoprotein originally identified as a tumor cell marker frequently up-regulated in human carcinomas (42). Its function remains largely unknown; however, it is phosphorylated by protein kinase C and cross-linking TACSTD2 with antibodies causes a transient increase in intracellular calcium levels, implying that it has a role in signal transduction (43). Regardless of its function in epithelial cells, it is frequently targeted for epigenetic silencing in glioma cell lines (five of six lines show TACSTD2 silencing); 68% of glioblastoma multiforme tumors show TACSTD2 hypermethylation; and TACSTD2 reexpression in LN-229 and U-87 MG cells suppressed cell growth at levels comparable with $p16^{INK4a}$. Calcium signaling is of paramount importance in normal brain function, and TACSTD2 may have roles in glial cells that differ from those in epithelial cells.

The tachykinins are a family of peptides that are traditionally considered to act as neurotransmitters. The *tachykinin 1* (*TAC1*) gene encodes several peptides by alternative splicing, including substance P, neurokinin A, neuropeptide K, and neuropeptide γ (44). Substance P has been the most thoroughly investigated and exerts its effects by binding to one of three known transmembrane G-protein–coupled tachykinin receptors (NK_1, NK_2, and NK_3). Malignant glial cells originating from astrocytes express functional NK receptors, and binding of substance P to glioma cells in culture and mouse xenograft models is a potent signal for them to release cytokines, such as interleukin-6 (IL-6), IL-8, and transforming growth factor-β (TGF-β), and increases their rate of proliferation (45). Despite the data showing that substance P may act as a mitogen, our data clearly indicate that TAC1 is down-regulated and hypermethylated in ~45% of the grade 3 to 4 gliomas. Interestingly, decreased levels of TAC1-encoded peptides like substance P in primary glioma samples has been reported (46). The exact role of TAC1 in primary human gliomas will, therefore, require additional study; however, the net effect of its reduced expression may depend on the local environment of the tumor. Release of certain cytokines may be immunologically unfavorable for the tumor (e.g., TGF-β), and it is unlikely that these effects would be observed in mouse xenografts (30).

Bcl-2-interacting killer (*BIK* or *NBK*) is a proapoptotic gene in the Bcl-2 BH3-only subfamily. Ectopic expression of BIK induces apoptosis in numerous tumor types, including colon, prostate, breast, melanoma, and glioma cells (31, 47, 48). BIK interacts with $Bcl\text{-}x_L$ and Bcl-2 and triggers apoptosis in a Bak-dependent manner (47). Importantly, BIK is frequently mutated in human peripheral B-cell lymphomas (49) and is within a region of chromosome 22q that undergoes loss of heterozygosity in ~30% of astrocytomas (50). These data, coupled with our findings that the BIK promoter is hypermethylated in ~30% of primary gliomas and that it is a potent suppressor of glioma cell growth in culture, strongly suggest that *BIK* is a tumor suppressor gene. Interestingly, we found that BIK hypermethylation was lower than the other genes studied, and in keeping with this idea, BIK can was inducible, albeit at lower levels, by HDAC inhibitor treatment alone. Therefore, it is likely that BIK is down-regulated by promoter hypermethylation or histone deacetylation, suggesting that our estimates for its frequency of down-regulation based on MSP will underestimate the total epigenetic silencing frequency (i.e., there may be a significant number of tumors with down-regulated BIK expression but no promoter hypermethylation). This notion is supported by a study that revealed that BIK was induced by HDAC inhibitors and antisense oligonucleotides against DNMT1 in a lung cancer cell line but the promoter of BIK was hypomethylated (24). BIK is therefore an attractive epigenetic target in glioma because it is a potent inducer of apoptosis, and because it can be reactivated by both DNMT and HDAC inhibitors.

TSPYL5 is a member of a gene family that includes TSPYL1, TSPYL2, TSPYL3, TSPYL4, and TSPYL6 (32). The function of the TSPYL genes is largely unknown, although clues come from the fact that they show homology to nucleosome assembly proteins (NAP) within their COOH-terminal regions. NAPs are involved in nucleosome assembly following DNA replication and act as histone chaperones, particularly for histones H2A and H2B (51). They also play roles in gene regulation because inactivation of the yeast *Nap1* gene altered the expression of about 10% of all yeast genes (52). TSPYL2 (CDA1 and CINAP) arrests cell growth and, like NAPs, binds core histones and facilitates chromatin assembly *in vitro* (53, 54). We found that TSPYL5 is hypermethylated in nearly 100% of the primary gliomas and was a marked suppressor of cell growth. Interestingly, TSPY also undergoes aberrant hypermethylation-mediated silencing in melanoma and increased methylation correlated with disease progression (55). Preliminary analysis of other TSPYL family members revealed that TSPYL2, TSPYL3, and TSPYL6 are also targeted for epigenetic silencing. Interestingly, a recent study showed that sudden infant death with dysgenesis of the testes (SIDDT) syndrome is due to mutations in the *TSPYL* (*TSPYL1*) gene. *TSPYL1* mutations in SIDDT resulted in truncated proteins lacking the region of homology with NAPs (56). Taken together, published studies and our results suggest that this family of genes is important for normal brain homeostasis by regulating chromatin structure and gene expression.

In summary, this work demonstrates the utility of the combined pharmacologic inhibition of DNA methylation/histone deacetylation and expression microarray approach to identify novel targets of aberrant epigenetic silencing in gliomas. Although considerable progress has been made in understanding the role of genetic alterations in glioma and in generating mouse models of this highly lethal tumor, there is still much to learn to understand their etiology and design new and more effective treatments. We have identified >30 novel targets of epigenetic silencing in the current article. Critical future studies will include examining a larger series of tumors to correlate silencing of these genes with tumor grade and patient survival. One or more of these genes may also provide a useful diagnostic or

prognostic marker for gliomas, particularly TSPYL5. Such biomarkers would be useful for confirming diagnosis by conventional methods, or for predicting initial disease or disease relapse after surgery (if hypermethylated alleles of these genes are detectable in blood or cerebrospinal fluid of cancer patients), before other methods are able to detect the presence of a tumor. Methylated alleles of one or more of the genes we have identified may also help to identify patients that will respond more favorably to a particular form of treatment.

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