Epigenetic Silencing of Death Receptor 4 Mediates Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand Resistance in Gliomas

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Epigenetic modifications contribute to tumorigenesis. Examples are epigenetically silenced tumor suppressor genes (1). However, the epigenome becomes crucial clinical relevance when epigenetically regulated proteins are identified that themselves constitute therapeutic targets or are responsible for the development of therapy resistance of the tumor. An example for the latter is O6-methylguanine DNA methyltransferase (MGMT) in glioma therapy. Epigenetic silencing of MGMT abolishes its capability of chemotherapeutic resistance and predicts therapeutic response to alkylating drugs in glioma therapy (2–4). Caspase-8 may exemplify a therapeutically targeted epigenetically silenced molecule in gliomas. A downstream target for tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)–mediated apoptosis, loss of caspase-8 expression, has been associated with epigenetic silencing and TRAIL resistance in these tumors (5). We were interested in identifying additional epigenetically silenced proteins or receptor molecules that are already targeted in glioma therapy. To address this, we implemented single-nucleotide polymorphism–based BeadArray technology. The GoldenGate Methylation Cancer Panel I is an excellent tool to screen for promoter hypermethylation in cancer-related genes (6, 7). The panel investigates a total of 1,505 CpG sites in 807 selected genes. These genes include oncogenes and tumor suppressor genes, previously reported differentially methylated or differentially expressed genes, genes involved in various signaling pathways, and genes responsible for DNA repair, cell cycle control, metastasis, differentiation, and apoptosis. Our analysis indicated

Abstract

Purpose: To identify and characterize epigenetically regulated genes able to predict sensitivity or resistance to currently tested chemotherapeutic agents in glioma therapy.

Experimental Design: We used methylation-sensitive BeadArray technology to identify novel epigenetically regulated genes associated with apoptosis and with potential therapeutic targets in glioma therapy. To elucidate the functional consequences of promoter methylation in the identified target death receptor 4 (DR4), we investigated tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)–mediated and anti-DR4–mediated apoptosis in glioma cell lines (U373 and A172) with loss of DR4 and one glioma cell line (LN18) with robust DR4 expression.

Results: In human astrocytic tumors, we detected DR4 promoter hypermethylation in 60% (n = 5) of diffuse astrocytomas WHO grade 2, in 75% (n = 8) of anaplastic astrocytomas WHO grade 3, and in 70% of glioblastomas WHO grade 4 (n = 33). DR4 is a cell surface protein restricted to glioma cells and is targeted by TRAIL. Glioma cell lines U373 and A172 harbored heavily methylated DR4 promoters, and 5-aza-2-deoxycytidine–mediated demethylation reconstituted DR4 expression in these cell lines. Functional knockdown of DR4 by DR4-specific small interfering RNA in TRAIL-sensitive glioma cell line LN18 significantly mitigated apoptosis induced by an agonistic anti-DR4 antibody. 5-Aza-2-deoxycytidine–mediated demethylation resulted in a functional reconstitution of DR4 on the cell surface of TRAIL-resistant glioma cell line U373 and sensitized U373 to TRAIL-mediated apoptosis. Suppression of DR4 by small interfering RNA in demethylated U373 successfully reestablished the TRAIL-resistant phenotype of U373.

Translational Relevance

Promising new cancer drugs are recombinant human tumor necrosis factor–related apoptosis-inducing ligand and the two newly designed monoclonal antibodies mapatumumab and lexatumumab that target the human death receptors 4 (DR4) and 5, respectively. All preferentially induce apoptosis in tumor cells and are currently being used under clinical trials in patients with advanced cancers. The main obstacle in TRAIL-based therapies, however, remains the resistance of cancer cells toward TRAIL and its analogues. We show that epigenetic silencing of DR4 is frequent and able to strongly attenuate TRAIL- or DR4-mediated apoptosis in gliomas. Our findings argue for mitigated sensitivity to TRAIL and resistance to mapatumumab in the majority of astrocytic tumors. However, a subgroup of gliomas still harbors a functional DR4 receptor predicted by an unmethylated DR4 promoter. Up-front testing for DR4 promoter methylation in tumor specimen may therefore help identify TRAIL- and mapatumumab-responsive cases among glioma patients.

Materials and Methods

Glioma samples and glioma cell lines. We investigated the methylation profile of 8 astrocytomas WHO grade 2 (AII), 8 anaplastic astrocytomas WHO grade 3 (AIII), and 15 GBM WHO grade 4 (GBM) using the GoldenGate Methylation Cancer Panel I (Illumina, Inc.).

An additional set of 20 unrelated GBMs was available for DR4 promoter methylation analysis by methylation-specific PCR (MSP). All tumors were classified and graded according to the 2007 WHO criteria (12). For functional DR4-related assays, we used the TRAIL-resistant GBM cell line U373 and the TRAIL-sensitive GBM cell line LN18. Cell lines LN18 and A172 were obtained from the American Type Culture Collection (distributed by LGC Standards GmbH). Cell line U373 was obtained from the European Collection of Animal Cell Cultures (distributed by Sigma-Aldrich Corp.).

Cell culture and reagents. Glioma cell lines were maintained in DMEM with 1-glutamine (Life Technologies, Inc., Invitrogen Corp.). The medium was supplemented with heat-inactivated 10% fetal bovine serum (Sigma-Aldrich) and 1% penicillin-streptomycin (Life Technologies, Invitrogen) and grown at 37 °C in a humidified atmosphere of 10% CO2/90% air.

DNA and RNA isolation. Total RNA was isolated from glioma cell lines LN18 and U373 using Trizol (Life Technologies, Invitrogen) according to the manufacturer’s instructions followed by postextraction cleanup using the RNaseasy Mini kit (Qiagen). Genomic DNA from glioma cell lines U373 and LN18, the snap-frozen glioma samples of the BeadArray analysis, and formalin-fixed, paraffin-embedded tissues was extracted using the DNA Isolation kit according to the manufacturer’s instructions (Genitra Systems). Genomic DNA from leukocytes and commercially available RNA and DNA from adult human brain (US Biological) were available as experimental controls.

DNA methylation profiling using universal BeadArrays. For DNA methylation analyses, we used the GoldenGate Methylation Cancer Panel I as previously described (6, 7). The panel has been developed to assay 1,505 CpG sites selected from 807 genes, which include oncogenes and tumor suppressor genes, previously reported differentially methylated or differentially expressed genes, genes involved in various signaling pathways, and genes responsible for DNA repair, cell cycle control, metastasis, differentiation, and apoptosis. Before BeadArray analysis, DNA isolated from the snap-frozen glioma samples (8 AII, 8 AIII, and 15 GBM) was bisulfite modified using EpiTect Bisulfite kit (Qiagen) following the manufacturer’s instructions.

Bisulfite sequencing and MSP. Bisulfite sequencing (BS) and MSP were done as previously described (13–15) and used as additional independent techniques for validation and investigation of the DR4 promoter region. MSP primers were designed based on DR4 promoter sequencing data and on previously published data on DR4 promoter methylation (16). Primer sequences are available on request. MSP of caspase-8 was done on the glioma specimens used for the BeadArray analysis (8 AII, 8 AIII, and 15 GBM) and the group of 20 unrelated GBM samples as previously reported (5).

5-aza-dC treatment of cells. Treatment with 5 μM/L 5-aza-dC (Sigma-Aldrich) was carried out for 96 h as previously described (13). All experiments were carried out in triplicates.

Reverse transcription-PCR analysis. For reverse transcription-PCR, first-strand cDNA was synthesized from 2 μg of DNA treated total RNA using the SuperScript First-Strand Synthesis System (Invitrogen) and oligo(dT) primers. Exon-overlapping CDNA-specific primers were designed for DR4. PCR was facilitated by AppliedIQ Gold PCR Master Mix (Applied Biosystems). Standard PCR amplification protocols were used with an initial denaturation and enzyme activation step of 95 °C for 15 min followed by 95 °C denaturation, individual annealing temperatures and 72 °C extension of 30 s each for 32 cycles, and a final extension at 72 °C for 10 min. β2-microglobulin (NM_000484) amplification was used as an internal control for CDNA quality and quantity. Amplified products were electrophoretically separated on 2% agarose gels and visualized with ethidium bromide.

Apoptosis induction. Apoptosis was induced by recombinant human TRAIL (rTRAIL)/Apo2L from PeproTech (distributed by Biozol) and agonistic anti-human DR4 antibody (agonistic anti-hDR4) obtained from R&D Systems.

Cancer Therapy: Preclinical

Clin Cancer Res 2009;15(17) September 1, 2009 5458 www.aacrjournals.org

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siRNA and transfection. Cells were seeded into six-well plates at 600,000 per well and transfected 4 h after seeding with siRNA at a final concentration of 10 nmol/L with 12 μL HiPerFect (Qiagen). All siRNAs were purchased from Sigma-Aldrich. Previously published sequences were used for synthesis of siGL3 (17), siDR4 (17), and siDR5 (18).

Fluorescence-activated cell sorting analysis

Determination of apoptotic cells by Annexin V staining. Apoptotic cells were assessed by Annexin V staining using Annexin V-FITC apoptosis detection kit (BD Biosciences) following the manufacturer's instructions and analyzed by flow cytometry using CellQuest (BD Biosciences). This kit detects phosphatidylserine on the outer surface of the cell membrane and assesses early stages of apoptosis. Flow cytometry was used to measure the percentage of positively stained cells. All experiments were done in duplicate, and results are given by the percentage of apoptotic cells.

Staining of cell surface of DR4. For detection of cell surface DR4, cells were harvested using Accutase (PAA), washed with cold PBS, and incubated either with the isotype control antibody (mouse IgG2b-phycoerythrin, R&D Systems) or with the DR4 antibody labeled with phycoerythrin (R&D Systems) for 30 min on ice. After washing with PBS, cells were subjected to flow cytometry. The specific fluorescence index 1 was calculated as the ratio of the geometric mean fluorescence values obtained with the isotype control antibody versus the DR4-specific antibody.

Western blot. Protein (20 μg) diluted in NuPAGE sample buffer and reducing reagent (Invitrogen) was denatured at 95°C for 5 min and electrophoretically separated on ready-to-use 4% to 12% SDS-PAGE (Invitrogen). Proteins were blotted onto nitrocellulose membranes at 1.5 mA/cm² for 1.5 h (Invitrogen). After blocking in 0.5 mol/L Tris-base (pH 7.4), 5% milk powder, 1.5 mol/L NaCl, and 0.05% Tween, the membranes were incubated with rabbit anti-human DR4 antibody (anti-hDR4) diluted 1:250 (Abcam), rabbit anti-human caspase-7 diluted 1:200, mouse anti-human caspase-8 diluted 1:200, rabbit anti-human poly(ADP-ribose) polymerase (PARP) diluted 1:250 (Cell Signaling Technology), and mouse anti-human tubulin diluted 1:2,000 (Sigma-Aldrich) overnight at 4°C. Staining with secondary horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies at dilutions of 1:10,000 or 1:2,000, respectively (Amersham Biosciences), was followed by immunodetection with Western Blotting Detection System ECL (Amersham Biosciences) and Lumigen TMA-6 (GE Healthcare).

Results

DNA methylation profiling using universal BeadArrays. Cutoffs to define unmethylated and methylated CpGs were selected in accordance to previous publications implementing identical
BeadArrays and the identical platform (GoldenGate Methylation Cancer Panel I; ref. 19). Accordingly, average β values of <0.2 defined unmethylated CpGs and average β values of >0.8 defined methylated CpGs. β Values of >0.8, suggesting a methylated DR4 promoter, were detected in 60% (n = 3/5) of AII, in 75% (n = 6/8) of AII, and in 62% (n = 8/13) of GBM (Supplementary Data 1).

BS and MSP. Excellent reproducibility of BeadArray results has been shown before (19). As a proof of principle, we validated the BeadArray results in selected GBMs by BS and MSP, confirming the previously observed high reproducibility. Exemplified BS and MSP data of validated gliomas are given in Fig. 1.

GBM cell lines U373 and A172 revealed a heavily biallelic hypermethylated DR4 promoter, whereas BS of the DR4 promoter in GBM cell line LN18 suggested a methylated DR4 promoter restricted to only one of the two alleles. This was shown by an equal distribution of either completely hypermethylated or completely nonmethylated clones in BS and a coexistence of methylated and nonmethylated bands in the MSP assay. Of note, the methylation status of the DR4 promoter was predicted by DR4 gene expression in all three cell lines. Results of BS and MSP in glioma cell lines U373, A172, and LN18 are provided in Fig. 1.

Finally, the DR4 promoter methylation status was investigated by MSP in a small series of 20 unrelated GBMs. Seventy-five percent (n = 15/20) revealed a hypermethylated DR4 promoter (Table 1).

In the glioma specimens used for the BeadArray analysis, caspase-8 methylation was detected in 43% (3 of 7) of AII, in 71% (5 of 7) of AII, and in 7% (1 of 20) of GBM. DR4/caspase-8 methylation was observed in 20% (1 of 5) of AII, in 57% (4 of 7) of AII, and in 8% (1 of 15) of GBM (Supplementary Data 1). In the group of 20 unrelated GBM, caspase-8 methylation by BS and DR4/caspase-8 methylation was detected in 10% (2 of 20) of the samples (Table 1). Caspase-8 was not methylated in all three glioma cell lines A172, U373, and LN18 (Supplementary Data 2A).

5-Aza-dC treatment of cells and DR4-related reverse transcription-PCR, Western blot, and fluorescence-activated cell sorting analyses in glioma cell lines A172, U373, and LN18. Loss of DR4 gene expression was found in A172 and U373 before demethylation treatment. LN18 was found to express DR4. Gene expression successfully predicted DR4 promoter methylation status in all three cell lines (Fig. 1). Western blot analysis revealed DR4 expression in LN18 as previously reported (20) and a loss of DR4 expression in glioma cell line U373. 5-Aza-dC–mediated demethylation was able to induce reexpression of DR4 both on RNA and protein level in glioma cell lines U373 and A172 (Fig. 2A and B). This indicates that 5-aza-dC–mediated demethylation succeeds in RNA and protein reexpression of the DR4 gene in glioma cell lines U373 and A172. In addition, these experiments link loss of RNA and protein expression of DR4 to a hypermethylated DR4 promoter in these cell lines.

Western blot analysis of caspase-8 in glioma cell lines A172, U373, and LN18. All three glioma cell lines revealed a robust caspase-8 protein expression (Supplementary Data 2B).

Expression of cell surface DR4. DR4 surface expression on U373 cells was assessed to confirm that induction of DR4 mRNA and protein by 5-aza-dC led to an increased expression of the DR4 receptor. U373 was treated with 5 μmol/L 5-aza-dC, and cell surface DR4 expression was assessed by flow cytometry.

5-Aza-dC increased the cell surface expression of DR4 in U373 (Fig. 2C). Thereby, fluorescence-activated cell sorting analysis not only showed a significant increase of DR4 expression on the cell surface following demethylation treatment but also indirectly showed an increased traffic of DR4 protein into the cell surface itself, the original site of DR4 function. These data strongly argue for the possibility of a functional reconstitution of the DR4 receptor in glioma cell lines with a hypermethylated DR4 promoter by 5-aza-dC–mediated demethylation. Of note, membrane expression of DR5 was not modulated by 5-aza-dC and the DR5 promoter was found to be unmethylated before demethylation treatment (Supplementary Data 2B).

Down-regulation of DR4 by specific siRNA mitigated TRAIL-induced apoptosis in LN18 glioma cells. To assess the importance of DR4 in death receptor–mediated apoptosis, we suppressed DR4 expression in LN18 glioma cells. LN18 glioma cells were transfected with either siRNA complexes targeting luciferase or DR4. DR4 protein expression was significantly attenuated in DR4-specific siRNA-transfected LN18 cells (Fig. 3B). Forty-eight hours after transfection, LN18 glioma cells were treated with 10 μg/mL of agonistic anti-hDR4 for 16 hours. Apoptosis was reduced from 75 ± 6% in LN18 cells transfected with luciferase-specific siRNA to 15 ± 3% in DR4-specific siRNA-transfected LN18 cells (P < 0.05; Fig. 3A). Apoptosis induction and activity was shown by the analysis of the protein expression of caspase-8 and of PARP and their respective cleaved fragments and by showing the cleaved fragment of caspase-7. Importantly, cleavage (activation) of initiator caspase-8, effector caspase-7, and the downstream cleavage target of effector caspase-3 was attenuated by the expression of siRNA directed against DR4.

<p>| Table 1. | MSP of DR4 and caspase-8 in 20 unrelated GBM specimens |</p>
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n (total) 20 20

Abbreviations: U, unmethylated; M, methylated.
caspases, PARP, was almost completely abrogated in DR4 siRNA-transfected LN18 cells (Fig. 3B).

5-Aza-dC potently sensitized U373 glioma cells to TRAIL-mediated apoptosis. We determined if 5-aza-dC is capable of sensitizing glioma cells to TRAIL-mediated apoptosis. Treatment with either 1,000 ng/mL TRAIL for 24 hours or 5-aza-dC for 96 hours did not significantly increase apoptotic cell death in U373 glioma cells. Importantly, 5-aza-dC resulted in an enhanced membranous expression of DR4 in U373 glioma cells.

Down-regulation of DR4 by specific siRNA mitigated 5-aza-dC–facilitated TRAIL-mediated apoptosis. To test the specific effect of DR4 on 5-aza-dC–facilitated TRAIL-mediated apoptosis, DR4 was silenced by specific siRNA in U373 glioma cells. Whereas DR4-specific siRNA dramatically suppressed 5-aza-dC–mediated up-regulation of DR4, nontargeted siRNA had no significant effect on DR4 expression in U373 glioma cells after treatment with 5-aza-dC. In addition, DR4-specific siRNA significantly attenuated 5-aza-dC–facilitated TRAIL-mediated apoptosis in U373 glioma cells. Forty-eight hours after transfection, U373 glioma cells were treated with 1,000 ng/mL TRAIL for 16 hours. Sixteen hours after treatment, apoptosis was reduced from 41 ± 4% in U373 cells transfected with luciferase-specific siRNA to 19 ± 3% in DR4-specific siRNA-transfected U373 cells (P < 0.05; Fig. 4C), indicating the importance of DR4 in 5-aza-dC–facilitated TRAIL-mediated apoptosis in U373 glioma cells primarily resistant toward TRAIL.

Fig. 2. Reexpression of DR4 after treatment with 5-aza-dC. A, DR4 mRNA levels in U373, A172, and LN18 glioma cells after demethylation. Note that 5-aza-dC induces reexpression of DR4 in U373 and A172. Robust DR4 expression remains unaffected in glioma cell line LN18. B, DR4 protein expression in U373, A172, and LN18 glioma cells after demethylation. Corresponding to A, 5-aza-dC induces DR4 expression in U373 and A172 and does not affect DR4 expression in LN18. C, DR4 cell surface expression in U373 cells after demethylation. Open histograms, following demethylation, U373 glioma cells were stained with phycoerythrin-conjugated monoclonal anti-DR4. Shadowed histograms, control cells stained with isotype-matched control IgG. A single experiment representative of two independent experiments with similar results. Right shift, presence of DR4 on cell surface.

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Fig. 3. Role of DR4 in TRAIL-mediated apoptosis in malignant glioma cells. A, top and bottom, Annexin V staining in LN18 cells 48 h after transfection with specific siRNA against luciferase or DR4 and 16 h after treatment with anti-hDR4. siLuc, specific siRNA targeted against luciferase; siDR4, specific siRNA targeted against DR4; anti-hDR4-0/10, 0 and 10 μg/mL of anti-DR4 (clone 69038). Experiments were done thrice. Asterisks (*) outline values that are different from the respective control. *, P < 0.05, t test. Middle, corresponding confocal cell culture images 16 h after treatment with anti-hDR4 in LN18 transfected either with siRNA against luciferase (siLuc) or against DR4 (siDR4). Note the dense apoptotic cell bodies in siLuc-transfected cells compared with mitigated apoptotic activity in siDR4-transfected cells, confirming fluorescence-activated cell sorting data. B, activation of initiator and effector caspases after treatment with anti-hDR4 in LN18 glioma transfected with either siRNA specific for luciferase or DR4. LN18 glioma cells were transfected as indicated in A, subsequently treated with 10 μg/mL anti-hDR4 for 16 h, and subjected to immunoblot analysis for DR4, caspase-8, PARP, cleaved caspase-7, and tubulin. Note that apoptosis was initiated only in LN18 cotransfected with siLuc and agonistic anti-hDR4, whereas cotransfection with siDR4 successfully blocked anti-hDR4-mediated apoptosis. Experiments were done thrice. Tubulin served as an internal loading and quality control.
Discussion

Despite aggressive treatment strategies, patients with GBM still have a dismal prognosis, which highlights the need for novel treatment approaches (8). One promising new cancer drug is rhTRAIL and two newly designed monoclonal antibodies targeting DR4 (mapatumumab; HGS-ETR1) and DR5 (lexatumumab; HGS-ETR2) because these agents have been shown to preferentially induce apoptosis in tumor cells (21). All three agents are implicated in current clinical trials in patients with advanced cancers (21). However, the main obstacle in TRAIL-based therapies remains the resistance of cancer cells toward TRAIL and its analogues. Therefore, identifying resistance mechanisms and predictors of response in these tumors remains a key issue in modern cancer diagnostics and therapeutic strategies. Among other possibilities, such predictive factors may be epigenetically regulated in a tumor-specific manner. In gliomas, MGMT has been identified as an example of an epigenetically regulated gene product of strong predictive value in combined radiotherapy and temozolomide therapy (2–4). To identify potential candidates, we used the BeadArray technology implementing the GoldenGate Methylation Cancer Panel I that allows comprehensive investigations of methylation patterns of cancer-related genes in tumors. It has a high reproducibility (19) and covers distinct pathways, including therapy-targeted end points such as apoptosis, and therefore may help identify epigenetically regulated key proteins within these pathways. This study is the first to provide evidence that TRAIL receptor 1 (DR4) is frequently silenced by promoter methylation in malignant gliomas, as 70% (23 of 33) of all GBM investigated here revealed a methylated DR4 promoter. DR4 promoter methylation was also found in high frequencies in low-grade and anaplastic astrocytomas of this study, suggesting it to be a common means of DR4 inactivation in all astrocytic tumors. However, only a limited number of low-grade and anaplastic astrocytomas were analyzed and extended data are needed to substantiate that initial observation. In line with our results are recent publications that report epigenetic silencing of DR4 in ovarian cancer (22), melanoma (16), and medulloblastoma (23). These and our data indicate that DR4 silencing may be common in a variety of tumors...
independent of germ layer provenance and may have a similar effect in all these tumors. Whereas DR4 promoter methylation was responsible for gene silencing in melanoma and ovarian cancer (16, 22), histone acetylation could be shown in medulloblastomas (23). Furthermore, it could be shown that silencing of DR4 contributes significantly to TRAIL resistance in melanomas (16). Our data strongly support this finding and for the first time show an equal function of DR4 in gliomas because down-regulation of DR4 by specific siRNA oligonucleotides significantly attenuated DR4-mediated apoptosis in LN18 glioma cells. In addition, we show that TRAIL resistance of glioma cell line U373 is due to loss of DR4 protein expression, mediated by DR4 promoter methylation. 5-aza-dC–mediated demethylation succeeded in a functional reconstitution of DR4 on the tumor cell surface and was able to make the TRAIL-resistant glioma cell line U373 susceptible to TRAIL-mediated apoptosis.

In addition, we specifically repressed the expression of DR4 in 5-aza-dC–treated U373 glioma cells by DR4-specific siRNA. Down-regulation of DR4 in these cells significantly mitigated 5-aza-dC/TRAIL–mediated apoptosis and reestablished the TRAIL-resistant phenotype of U373 glioma cells, underscoring the importance of DR4 in TRAIL-mediated apoptosis. This result is of particular importance because DR4 expression was necessary to initiate 5-aza-dC–facilitated TRAIL-mediated apoptosis and other TRAIL receptors (i.e., DR5) were not sufficient to compensate for the loss of DR4, suggesting a crucial role for DR4 in TRAIL-mediated apoptosis in malignant glioma. These results strongly argue for functional relevance of epigenetic silencing of DR4 in gliomas. Most recently, it has been reported that a single amino acid mutation at the position of glycine 131 to lysine or arginine in wild-type rhTRAIL induces higher in vitro levels of apoptosis in cancer cells responsive only to DR4 (24). This allows two interpretations. First, wild-type rhTRAIL per se may have a lower affinity to DR4 than to DR5 so that, secondly, we may have seen an even stronger effect of DR4 reexpression in TRAIL-mediated apoptosis with rhTRAIL harboring that single mutation. Cell sensitivity to TRAIL can be affected by TRAIL receptor expression at the cell membrane, DR4/DR5 ratio, and functionality of TRAIL receptors. Several additional intracellular factors leading to TRAIL resistance downstream of the receptor molecules have been identified. The caspase-8/c-FLIP ratio may be affected, leading to changes in caspase-8 or c-FLIP expression levels. Caspase-8 and caspase-10 have been found lost due to gene mutations or promoter methylation. In addition, degrada-

In particular, it did not address caspase-8 gene methylation. In several tumor cell lines and primary tumors, caspase-8 gene methylation has indeed been reported to underlie down-regulation of caspase-8 and TRAIL resistance (27). In our series, we observed a higher frequency of caspase-8 gene methylation in AII and AIII compared with GBM (Supplementary Data 1). These findings are in line with observations of others (28). More recently, caspase-8 gene methylation was investigated in relapsed GBM (5). In line with our observation of a low rate of caspase-8 gene methylation in GBM, none of the primary GBM samples was methylated for caspase-8. However, 43.8% (7 of 16) of the relapsed GBM revealed caspase-8 gene methylation. This suggests that caspase-8 gene methylation is a differential feature of recurrent GBM (5). In addition, the rate of DR4/ caspase-8 comethylation was higher in low-grade and anaplastic tumors than in GBM (Supplementary Data 1). These and our data together with the observation that reversal of caspase-8 gene methylation by 5-aza-dC was able to restore apoptosis sensitivity of affected tumor cell lines, including malignant gliomas (27), may argue for caspase-8 gene methylation to act in part as a contributing factor to deregulated apoptosis mechanisms in gliomas. In an effort to exclude the possibility of caspase-8–mediated TRAIL resistance in our cell lines, we analyzed the protein expression of caspase-8 by Western blot and its gene methylation status by MSP. We observed an unaltered expression of caspase-8 and unmethylated caspase-8 promoter in all presented cell lines (Supplementary Data 2A). These data strongly suggest that the changes we saw in TRAIL sensitivity after demethylation was caspase-8 independent and DR4 specific.

Glioma cell line LN18 harbored one allele with a completely unmethylated DR4 promoter. It showed strong DR4 protein expression on its cell surface (20) and was highly susceptible to apoptosis induced by an agonistic anti-hDR4 antibody. These data imply that a subset of gliomas with an unmethylated DR4 promoter may be susceptible to anti-DR4–directed therapy. Most recently, two clinical phase II trials implementing mapatumumab have been completed enrolling patients with either relapsed or refractory non–small cell lung cancer or patients with relapsed or refractory non–Hodgkin’s lymphoma. The respective clinicalTrials.gov identifier numbers are NCT00092924 and NCT00094848. In gliomas, the combination of diffusely infiltrating tumor growth and cell surface expression of DR4/DR5 receptors confined to tumor cells may make agonist anti-DR4 antibodies therapeutically attractive in the near future. Our data suggest that epigenetic silencing of DR4 in gliomas may predict anti-DR4 resistance. Our data warrant epigenetic testing in therapeutic strategies that target DR4 and may prove a diagnostic marker for response in these tumors.

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

References
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doi:10.1158/1078-0432.CCR-09-1125

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