Tumor-Suppressive miR148a Is Silenced by CpG Island Hypermethylation in IDH1-Mutant Gliomas

Sichen Li1, Reshmi Chowdhury1, Fei Liu1, Arthur P. Chou2, Tie Li1, Reema R. Mody1, Jerry J. Lou1, Weidong Chen1, Jean Reiss3, Horacio Soto2, Robert Prins2, Linda M. Liau2, Paul S. Mischel4, Phioanh L. Nghiemphu1, William H. Yong3, Timothy F. Cloughesy1, and Albert Lai1

Introduction

Diffuse gliomas represent the most common type of primary brain cancer in adults and remain incurable (1). Diffuse gliomas are diagnosed histologically according to the World Health Organization (WHO) classification system; however, a detailed molecular framework can now be overlaid on this existing classification system (2). In particular, mutation of either the isocitrate dehydrogenase 1 or 2 (IDH1/2) gene has now been accepted as an initiating step and molecular marker for gliomas arising along the secondary glioblastoma/oligodendroglioma pathway (2–4).

Several DNA methylation studies have described a coordinated CpG island hypermethylation signature (glioma-CpG island methylator phenotype, G-CIMP) in gliomas harboring the IDH1/2MUT (5, 6); and accumulating evidence suggests that aberrant IDH1/2MUT protein function contributes to the DNA hypermethylation pattern observed in G-CIMP (7, 8). Through methylation and silencing of potential tumor-suppressive genes, the composition and scope of CpG islands/genes within G-CIMP are thought to provide important clues to understanding the initiation and progression of tumors along the secondary glioblastoma/oligodendroglioma pathway (5, 6). The clinical relevance is that reexpression of these silenced genes may be an effective strategy to control these tumors. However, major barriers impeding progress in this direction include both lack of consensus on the exact composition of G-CIMP CpG islands/genes, and lack of detailed functional studies in glioma demonstrating tumor-suppressive function of these genes (9).

To address the lack of consensus on G-CIMP composition, we have recently published the first single base resolution methylation profiling characterization of G-CIMP...
**Translational Relevance**

_IDH1/2-mutant gliomas harbor a distinct glioma-CpG island methylation phenotype (G-CIMP) hypothesized to play a role in the development and progression of secondary pathway gliomas by silencing tumor-suppressive genes. Although various protein-coding genes have been characterized as G-CIMP members, noncoding microRNAs (miRNA) have not yet been recognized within G-CIMP. More importantly, the functional validation of the G-CIMP genes, whether coding or noncoding, contributing to glioma progression and DNA methylation deregulation is still lacking. The identification and characterization of hypermethylation silenced miRNAs within G-CIMP could therefore contribute to a better understanding of the underlying mechanisms of the development and progression of secondary pathway gliomas, and provide a pharmacologic rationale for the potential use of demethylating agents and synthetic miR148a mimics as approaches for the treatment of IDH1-mutant gliomas._

**Materials and Methods**

**Cell culture and treatments**

Glioma cell lines and HEK293T cell line were a generous gift from Dr. Paul Mischel (University of California San Diego, La Jolla, CA) and originally obtained from the ATCC. HT1080 cell line was purchased from the ATCC (CCL-121). Human neuron stem cells (hNSC) were obtained from Dr. Harley Kornblum [University of California Los Angeles (UCLA), Los Angeles, CA). Glioma cell lines were maintained in DMEM/F12 cell culture medium with 10% FBS and penicillin/streptomycin (Invitrogen). HEK293T cell line and HT1080 cell line were maintained in DMEM cell culture medium with 10% FBS and penicillin/streptomycin. hNSCs were maintained in neurobasal medium supplied with EGF (50 ng/mL), basic fibroblast growth factor (bFGF; 20 ng/mL). B27, and penicillin/streptomycin (Invitrogen). All cells were cultured at 37°C and 5% CO2 in tissue culture incubator.

**Patient cohorts and tumor specimens**

A total of 324 frozen and formalin fixed paraffin-embedded (FFPE) tissue specimens were obtained from the UCLA Brain Tumor Translational Resource. Remnant human brain tumor samples were collected from patients undergoing surgical resection and who provided written informed consent. The collection of human brain tumor samples was approved by the UCLA Institutional Review Board. _IDH1_ was sequenced on all samples and _IDH2_ was sequenced on selected _IDH1_ samples (including all samples used for RRBS analysis and those with miR148a methylation).

**Constructs**

Retrovirus constructs for _IDH1_ and _IDH1_R132H expression (pLPCX, pLPCX-IDH1-WT, and pLPCX-IDH1-R132H) were generated as previously reported (23). Lentivirus constructs for miR148a expression (pMIF-GFP-zeo-mir148a and pMIF-GFP-zeo) were a generous gift from Dr. Olga Aprelikova (Center for Cancer Research, National Cancer Institute, MD, U.S.). Promoter reporter vector pIS0-DNMT1-3′-UTR-WT and pIS0-DNMT1-3′-UTR-MUT were generated by cloning 3′-untranslated region (UTR) sequence of _DNMT1_ gene using the following primers: forward, 5′-GTAATTCAGGCATCTTCTGCGCTCCGTC-CACCCCG-3′; reverse, 5′-CCGCCCGAGCTCAGTTGATGGTGTGTTATGAGAGATTTG-3′. For three sites specific mutagenesis of the _DNMT1_ reporter vector, the following primers were used: forward, 5′-CTGGGACACAGGAAATCCCAAC-AcGaaGATGTTCTGTGTTTTACAC-3′; reverse, 5′-TCAATcG-TGTGTGAGATTCCTTGTCGACAAACAGGGTTGACG-3′ (mutated sites are indicated with lower case).

**Massively parallel RRBS and _IDH1_ and _IDH2_ sequencing**

RRBS and _IDH1_ and _IDH2_ sequencing were done using the protocol published previously (6).

**Analysis of methylation and gene expression in The Cancer Genome Atlas dataset**

miRNA expression data, measured using the Agilent Sureprint 8 × 15k Human miRNA microarray (Agilent), were obtained for 145 The Cancer Genome Atlas (TCGA) samples. Level 3 expression data, including normalized expression signal per miRNA per sample, were downloaded directly from the TCGA data portal (http://tcga-data.nci.nih.gov/tcga/).

**Bisulfite conversion of genomic DNA, target bisulfite sequencing, and methylation-specific PCR**

Bisulfite conversion of genomic DNA and standard bisulfite sequencing (BiSEQ) using a nested PCR protocol
was used as described previously (6). The methylation status of the miR148a promoter CpG island (region 1: chr7:25991097-25991208; region 2: chr7: 25990178-25990308) was assessed using primer sets shown in Supplementary Table S10. The sequence of each sample was reviewed using Chromas Lite 2.33 (Technelysium Pty Ltd.), and CpG sites exhibiting a substantial signal for C (as compared with T) were considered methylated. The samples that had less than three methylated CpG sites were considered unmethylated (Unmethyl.), otherwise considered methylated (Methyl.). Methylation-specific PCR (MSP) was performed using a nested PCR protocol as previously described (24). The methylation-specific primer set was shown in Supplementary Table S11.

RNA isolation and quantitative reverse transcription PCR

Total RNA was isolated using TRizol reagent according to the manufacturer’s guidelines (Invitrogen). The integrity of total RNA was determined by 1% agarose gel electrophoresis. For RT-PCR, reverse transcription of total RNA was performed by the Reverse Transcription System (Promega) or the TaqMan Reverse Transcription system (Applied Biosystems) according to the manufacturer’s protocol. The qPCR was carried out using the LightCycler 480 System (Roche) with the universal SYBR Green PCR master mix (Roche) by using b-actin as internal control. All results from three independent experiments are presented as mean ± SEM (n = 3).

Analysis of miRNA expression using the TaqMan Reverse Transcription PCR

Expression of mature miR148a was analyzed using the TaqMan MicroRNA Assays (Applied Biosystems). Expression of RN16B (Applied Biosystems) was used as an endogenous control. miR148a expression was measured relative to RN16b (internal control) and quantified by the relative Ct method (2^ΔΔCt). All the results are from three independent experiments carried out in duplicate. Two commercially available normal brain cDNA libraries were used as normal control (Invitrogen; Biochain). The TaqMan qPCR was carried out using the LightCycler 480 System (Roche) with the TaqMan universal PCR master mix (Applied Biosystems). All results from three independent experiments were performed in duplicate are presented as mean ± SEM (n = 3).

Western blot analysis

Western blot analysis was carried out using standard methods. The following primary antibodies were used: anti-DNMT1 (Abcam) at 1:800 dilution; anti-0-tubulin (Sigma) at 1:2,000 dilution; anti-FLAG (Sigma) at 1:2,000 dilution; anti-IDH1 (Santa Cruz Biotechnology) at 1:750 dilution; and anti-IDH1R132H (Dianova) at 1:750. The following secondary antibodies were used: horseradish peroxidase (HRP)–conjugated goat anti-rabbit (1:4,000) IgG (Santa Cruz Biotechnology), HRP-conjugated donkey anti-goat (1:8,000) IgG (Santa Cruz Biotechnology), and HRP-conjugated goat anti-mouse IgG (1:10 000; Jackson ImmunoResearch). An enhanced chemiluminescence detection kit (Pierce) was used for the detection of HRP. Densitometry was performed with Gel-Pro Analyzer 4.0 software (Media Cybernetics).

Luciferase reporter assay

After closely examination of miR148a precursor gene and analysis of miR148a promoter activity by using bioinformatics method, we have identified that miR148a–associated CpG island is located at −407 to −1716 upstream of miR148a precursor region, and it is overlapped with the predicted transcription start site (TSS; −1112) as shown in Fig. 3D. Of note, 1.623-kb DNA fragment localized at the upstream of predicted miR148a TSSs (−1038 to −2661) was cloned into pGL4.17 vector (pGL4.17-miR148a-P). Wild-type (WT) or mutant 3′-UTR of DNMT1 were cloned into Firefly luciferase reporter pl50 vector (Addgene plasmid; Cat no., 12178). Renilla luciferase vector pRL-SV40 (Promega) was cotransfected in each experiment as an internal control. Transfection was carried out by using X-Treme GENE HP Transfection Reagent (Roche) according to the manufacturer’s protocol. Firefly and Renilla luciferase activity was measured by the Dual-Luciferase Assay Kit (Promega) on a Wallac Victor2 plate reader (PerkinElmer) and data were normalized to Renilla activity 24 hours after the transfection. All results from three independent experiments were performed in six repeats and are presented as mean ± SEM (n = 3).

Stably overexpression of IDH1-mutant protein in HEK293T cells

Isogenic HEK293T cell lines stably overexpressing mutant IDH1 (R132H), WT IDH1, and empty vector (EV) were generated by using retrovirus constructs for IDH1WT or IDH1R132HMUT as described previously (23).

Transient and stable transfection of miR148a

miR148a mimics and negative control mimics were purchased from Ambion and transient transfection (30 nmol/L miRNA mimics) was performed using siPORT NeoFX transfection region according to the manufacturer’s instructions. Stable overexpression of miR148a was performed using Lentivirus constructs for miR148a. The packaging of lentivirus was conducted by using Lentivirus Expression System (SBI) according to the manufacturer’s instructions.

DNMT1 knockdown

Silencer select DNMT1 siRNA (10 nmol/L) and negative control siRNA (10 nmol/L) were purchased from Ambion and transfected by using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions at days 1, 3, and 5 after seeding into 24-well plates. Total RNA was isolated 48 hours after the last transfection.

MTT assay

Cells were seeded into 24-well plates. Cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,
5-diphenyltetrazolium bromide (Sigma) assay as previously described (23). Absorbance at 535 nm was measured on a Wallac Victor2 plate reader (PerkinElmer) with a background reference filter at 660 nm. All results from three independent experiments were performed in six repeats and are presented as mean ± SEM (n = 3).

**Soft agar assay**

Growth in soft agar was measured by colony assay as previously described (23). Briefly, 2-mL under-layers of 0.6% agar medium was prepared in 6-well plates by combining equal volumes of 1.2% Noble agar (Fisher) and DMEM with 20% fetal bovine serum. Cells (2 × 10^5) were plated in 0.3% agar medium and cultured for 2 to 3 weeks. Colonies were then photographed, and colonies larger than 50 μm in diameter were counted in five random microscopic fields and tabulated. All results from three independent experiments were performed in three repeats and are presented as mean ± SEM (n = 3).

**Colony formation assay**

One thousand cells were seeded onto 60-mm dishes and incubated in the tissue culture incubator for 14 days. Cells were fixed with methanol and stained with 0.25% crystal violet. Colonies containing >50 cells were counted under a dissecting microscope. The results are reported as a percentage of the colonies in untreated cultures of each corresponding clone. All results from three independent experiments were performed in six repeats and are presented as mean ± SEM (n = 3).

**Wound-healing assay**

Wound-healing assay was performed as previously described (23), and cells were plated in 6-well plates coated with 0.1% gelatin to create a confluent monolayer. The scratch wound was observed using contrast microscopy (Olympus, IX41), and images were taken at 0, 6, 9, 12, 24, and 36 hours after the initial scratch. Scratch wound distance was quantitated using Adobe Photoshop software. All results from three independent experiments were performed in six repeats and are presented as mean ± SEM (n = 3).

**FACS analysis**

Cells were dissociated with Trypsin and washed twice in ice-cold PBS before fixed with 70% ethanol/PBS at 4°C overnight. Cells were washed twice in ice-cold PBS and resuspended in staining solution (0.1% Triton X-100, 0.01% propidium iodide, and 0.002% Ribonuclease A in PBS) for at least 30 minutes on ice. Cells were passed through a filter and placed in a tube before measuring with FACSscan analyzer (Becton Dickinson) in Flow Cytometry Core Facility at UCLA. All results from three independent experiments were performed in six repeats and are presented as mean ± SEM (n = 3).

**Xenograft model**

All animal experiments were approved by the UCLA Institutional Animal Care and Use Committee. Two groups of male NOD/SCID mice (6–8 weeks old) were used for either subcutaneous injection or intracranial injection as follows: (i) U251-EV cells (EV control; n = 6); and (ii) U251-miR148a cells (stably transfected with miR148a; n = 6). For the subcutaneous injection, mice were anesthetized and injected with 10^6 glioma cells in 100 μL 10% matrix gel/PBS into the right flank. Tumor volume was monitored over time as determined from calculation (w × l × thickness/6) using two perpendicular measurements with a caliper. Mice were killed 30 days after injection, and tumors from both groups were excised and weighed. The mean volume or tumor mass SEM was calculated. For the stereotactic intracranial injection, the surgical site was shaved and prepared with 70% ethyl alcohol. A midline incision was made and a 1-mm diameter right parietal burr hole, centered 2 mm posterior to the coronal suture and 2 mm lateral to the sagittal suture, was drilled. Mice were placed in a stereotactic frame and 2.5 × 10^5 glioma cells in 2-μL 10% matrix gel/PBS were intracranially injected with a 26-gauge needle at a depth of 3 mm. The needle was removed and the skin incision was closed by holding the edges with forceps and applying one clip. Mice were monitored daily and killed 60 days after injection. Survival was estimated using the Kaplan–Meier method, and comparisons between groups were performed using the log-rank test. The presence of macroscopic brain metastases was analyzed by H&E tissue staining.

**Statistical analysis**

Differentially methylated CpG islands located in miRNA promoter regions were identified by performing the Student t test between IDH1/2^MUT and IDH1/2^WT samples. To reduce the number of false-positive results to a minimum threshold of 0.2 was set for the difference of means between IDH1/2^MUT and IDH1/2^WT samples. In addition, to control for multiple testing, we set a significance threshold of Q < 0.05, evaluated as per Storey (25). All other quantitative comparisons were done using the Student t test, the Wilcoxon test (nonparametric paired analysis), and the Mann–Whitney U test (nonpaired analysis). Qualitative variables were analyzed using the χ² test and the Fisher exact test. All tests were two-sided, and a P value of less than 0.05 was considered statistically significant. The other in vitro and in vivo data were analyzed using the GraphPad Prism 5.0 statistical software. Quantitative variables were analyzed using the Student t test, Wilcoxon test (nonparametric paired analysis), and Mann–Whitney U test (nonpaired analysis). Qualitative variables were analyzed using the χ² test or the Fisher exact test. A two-sided P value of <0.05 was regarded as significant.

**Results**

**Identification of hypermethylated CpG islands associated with miRNAs in gliomas harboring the IDH1 mutation**

To identify hypermethylated Cpg islands proximally associated with miRNAs in IDH1/2^MUT gliomas, we performed...
RRBS as previously described (6) to profile methylomes of 11 IDH1/2WT and 20 IDH1MUT patient glioma tissues (Fig. 1A). This included five IDH1/2WT and five IDH1MUT samples as previously reported (6). The clinical characteristics of these 31 patients are listed in Supplementary Table S1, and the coverage details of the RRBS methylation data are shown in Supplementary Table S2. Overall, we generated methylation coverage details of the RRBS methylation data are shown in Supplementary Table S3. Among them, the miR148a associated CpG island spans a 1.3-kb region between −407 to −1716 upstream of the pre-miR148a region.

CpG islands within 5,000 base pairs of 430 miRNAs and 182 CpG islands within 500 base pairs of 198 miRNA (Supplementary Table S4). Among them, the miR148a expression level was significantly higher in IDH1MUT gliomas compared to IDH1WT gliomas (Fig. 1B; Table 1; Supplementary Table S4). Among them, the miR148a associated CpG island spans a 1.3-kb region between −407 to −1716 upstream of the pre-miR148a region.

![Diagram](image-url)

**Figure 1.** Discovery of hypermethylated miRNAs in G-CIMP. A, a schematic strategy used to identify target miRNAs in G-CIMP. B, unsupervised hierarchical clustering of differentially methylated CpG islands (P < 0.05, unpaired t test) that were identified by comparing IDH1MUT (MUT) and IDH1WT (WT) glioma patient samples using RRBS. All CpG islands within 5,000 bp of pre-miRNA regions are shown. C, methylation profile of the miR148a-associated CpG island via RRBS. Top, map of the miR148a CpG island (chr7:25990013-259991319), position of pre-miR148a region (black box, chr7:25998539-25998606), predicted putative TSSs, and PCR products used for BiSEQ in regions 1 and 2 (black arrow); bottom, representative CpG island methylation pattern of IDH1WT gliomas or IDH1MUT gliomas determined by RRBS. D, differential expression data for miR148a in IDH1WT and IDH1MUT GBM from the TCGA dataset. E, the TaqMan qPCR analysis of relative miR148a expression in IDH1WT and IDH1MUT glioma tissue samples from validation cohort. Data are standardized to the mean value for IDH1WT samples, which was set as 100%.

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Integrated analysis of expression and methylation identifies miR148a as a hypermethylated and silenced miRNA in IDH1MUT gliomas

On the basis of the notion that functionally important hypermethylated miRNAs should display decreased expression via epigenetic silencing, we integrated miRNA expression data from the TCGA (http://tcga-data.nci.nih.gov/tcga/) with our RRBS methylation data to determine methylated miRNAs with downregulated expression. To do so, we calculated mean miRNA expression levels in IDH1MUT (n = 22) versus IDH1WT (n = 115) tumors (n.b. IDH2 status not available). The TCGA miRNA microarray analysis provided data for two of the eight miRNA candidates with hypermethylated Cpg islands within 500 base pairs of their precursor region (Table 1); therefore, we randomly selected three of the remaining six possible miRNA candidates (miR935, miR3131, and miR4710) and performed expression analysis using the TaqMan qPCR on a set of glioma tissue samples. Among these five candidate miRNAs with expression data, only miR148a showed significantly reduced expression in IDH1MUT tumors compared with IDH1WT tumors (Table 1 and Fig. 1D and E).

To confirm the DNA methylation status of the miR148a-associated Cpg island in a larger validation set, we performed direct targeted BisEQ in 17 autopsy normal brain tissue samples, 219 IDH1WT and 72 IDH1/2MUT glioma tissue samples (including two IDH2MUT samples; Supplementary Table S5). The miR148a-associated Cpg island is located on chromosome 7 (chr7: 25990012-25991320; ref. 28), 407 base pairs upstream of pre-miR148a region (−407 to −1716), and contains 123 Cpg sites and two predicted TSSs (TSS1: −213; TSS2: −1038; http://www.cbs.dtu.dk/services/Promoter/; ref. 28). Approximately 90 of 123 Cpg sites were covered by RRBS (Supplementary Fig. S1). The representative Cpg island methylation pattern of miR148a characterized by RRBS, along with the localization of predicted TSSs and PCR products for BisEQ, is shown in Fig. 1C. Overall, we found that miR148a was hypermethylated in region 1 in 0% (0 out of 17) normal brain tissues samples, 5.4% (12 out 219) IDH1WT glioma tissue samples, and 97.2% (70 out of 72) IDH1/2MUT glioma tissue samples (Supplementary Table S5). To validate that differential methylation was also present in region 2 of the miR148a Cpg island, we performed BisEQ in region 2 on a subset of patient tissue samples and obtained similar results as in region 1 (Supplementary Table S6). These results show that miR148a promoter hypermethylation is associated with IDH1/2 mutation within all grades and histology.

To confirm that methylation was associated with decreased expression in our samples as observed in the TCGA dataset, we analyzed the expression of miR148a in the available frozen glioma tissues within the BisEQ validation set by using the TaqMan qPCR. We found that miR148a expression level was downregulated in IDH1MUT gliomas in tight correlation with its Cpg island methylation status (Fig. 1E).

miR148a methylation is prognostic of increased survival in malignant gliomas

To determine whether miR148a methylation can be used as a prognostic biomarker, we correlated miR148a methylation with overall survival (OS) in the 224 patients with primary GBM and 42 patients with grade 3 glioma in our cohort who had treatment-naive tumor samples (Supplementary Table S7). In the patients with glioblastoma (GBM), miR148a-unmethylated patients (n = 195) had decreased median OS of 17.0 months versus 36.3 months (log-rank, P = 0.01; Fig. 2A). By univariate Cox analysis, the patients with miR148a-unmethylated GBM had a hazard ratio (HR) = 1.82 [confidence interval (95% CI), 1.14–2.89]. Table 1. Hypermethylated CpG islands at least partially within 500 bp of miRNAs in IDH1MUT versus IDH1/2WT gliomas (G-CIMP)

<table>
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<th>TCGA gene expression</th>
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</table>

Abbreviation: NA, data not available.

NOTE: As described in the Results, we did not perform TaqMan qPCR on miR2682, miR4520b, miR4520a; miR935, miR2682, miR4520b, miR4520a, miR3131, miR4710 were not covered by TCGA database.
2.90; \( P_{\text{Cox}} = 0.011 \); Supplementary Table S8). In the patients with grade 3 glioma, patients with miR148a-unmethylated \((n = 12)\) had a decreased median OS of 21.6 versus 81.3 months \((\log, \text{rank}, \text{log-rank}, P = 0.002; \text{Fig. 2B})\); for the patients with miR148a-unmethylated, the HR was 4.28 \((CI, 1.58\text{--}11.59; \ P_{\text{Cox}} = 0.004; \text{Supplementary Table S8})\). Using multivariate analysis with age, sex, performance status, and extent of resection, miR148a-unmethylated was confirmed as an independent prognostic marker in grade 3 glioma \((HR, 5.83; CI, 1.47\text{--}23.08; \ P_{\text{Cox}} = 0.012)\) and tended toward being an independent prognostic marker in GBM \((HR, 1.59; CI, 0.98\text{--}2.6; \ P_{\text{Cox}} = 0.059; \text{Supplementary Table S8})\). Similar to our previous finding, IDH1/2 mutational status was an independent prognostic marker in an overlapping dataset \((\text{Supplementary Fig. S2}; \text{ref. 6})\). However, when patients with miR148a-methylated were stratified by IDH1/2 status, the methylated IDH1/2 WT patients \((\text{GBM: } n = 10; \text{grade 3: } n = 2)\) had similar poor survival to miR148a-unmethylated IDH1/2 WT patients \((\text{data not shown})\). This suggests that miR148a methylation is unlikely to add to IDH1/2 mutational status in prognostication.\n
Downregulated miR148a associated with promoter CpG island hypermethylation can be reexpressed by either pharmacologic or genetic demethylation

To provide additional evidence that CpG island promoter methylation of miR148a is responsible for silenced expression, we evaluated methylation and expression of miR148a in a variety of cell lines including glioma cell lines \((\text{U251, LN18, T98G, and U87})\), hNSCs, human HT1080 fibrosarcoma cells \((IDH1^{\text{WT}}, \text{IDH1}^{\text{MUT}})\), and human HEK293T epithelia kidney cells. We found that miR148a was hypermethylated in all of the glioma and HT1080 cells, and hypomethylated in hNSC and HEK293T cells. These results indicate that miR148a hypermethylation developed in the IDH1/2 WT glioma cell lines by mechanisms independent of IDH1/2-mutant protein expression. Next, we analyzed the expression of miR148a in the cell lines by using the TaqMan RT-qPCR, and found, as expected, that there was silencing of expression in the methylated cell lines \((\text{Fig. 3A})\). To determine whether pharmacologic demethylation could restore miR148a expression in hypermethylated cell lines, we treated the cells with 5-aza-CdR and observed upregulation of miR148a in glioma cell lines with hypermethylated miR148a, but not in HEK293T or hNSC cells with hypomethylated miR148a \((\text{Fig. 3B})\). To confirm that 5-aza-CdR treatment resulted in demethylation, we used the same conditions as in Fig. 3B \((5 \mu \text{mol/L 5-aza-CdR treatment for 4 days})\) and found that there was qualitatively a slight decrease in methylation using MSP. We followed up this experiment using prolonged low-dose 5-aza-CdR treatment based on published reports \((29)\) and found that 0.625 \(\mu \text{mol/L 5-aza-CdR (9 days treatment) resulted in dramatically decreased demethylation using both BiSEQ (Supplementary Fig. S3A) and MSP (Supplementary Fig. S3B). Significant increase of miR148a expression in the long-term 5-aza-CdR–treated U251 cells was also detected by the TaqMan qPCR (Supplementary Fig. S3C). These results provide evidence that 5-aza-CdR increases expression of miR148a via demethylation but do not rule out the possibility of methylation-independent effects of 5-aza-CdR. Consistent with these results, we found that knockdown of DNA (cytosine-5-) methyltransferase 1 \((DNMT1)\) using siRNA also resulted in upregulated expression of miR148a in U251 glioma cells \((\text{Fig. 3C and Supplementary Fig. S4})\). Finally, using a luciferase reporter assay conducted in HEK293T, we confirmed that a 1,623-bp long fragment upstream of pre-miR148a gene \((-1038\text{--}2661)\) overlapping the CpG island \((-407\text{--}1716)\) possesses promoter activity \((\text{Fig. 3D})\). Taken together, these results strongly suggest that the expression of miR148a can be silenced through promoter CpG island methylation in glioma.\n
Overexpression of IDH1-mutant protein in HEK293T cells results in the hypermethylation and silencing of miR148a

As shown in Fig. 3A, all glioma-derived cell lines demonstrate miR148a hypermethylation, and therefore cannot be used to demonstrate causality between IDH1 mutation and miR148a hypermethylation. Therefore, to provide

Figure 2. The methylation status of miR148a is prognostic in GBM and grade 3 gliomas. A, Kaplan–Meier OS analysis of patients with GBM in the validation cohort \((n = 224)\). Survival among miR148a-methylated \((n = 29, \text{solid line})\) and miR148a-unmethylated \((n = 195, \text{dotted line})\) patients is shown. B, Kaplan–Meier OS analysis of patients with grade 3 glioma in the validation cohort \((n = 42, \text{solid line})\). Survival among miR148a-methylated \((n = 30)\) and miR148a-unmethylated \((n = 12, \text{dotted line})\) patients is shown.
evidence linking hypermethylation of miR148a to IDH1-mutant protein expression, we generated isogenic HEK293T cell lines stably overexpressing mutant IDH1 (R132H), WT IDH1, and EV (Supplementary Fig. S5). By monitoring methylation longitudinally using targeted BiSEQ, we detected miR148a promoter CpG island hypermethylation in HEK293T-IDH1R132HMUT after passage 17 but not in the parent HEK293T cells, HEK293T-EV cells, or HEK293T-IDH1WT cells assayed in parallel at the same passage (Fig. 3E). The TaqMan RT-PCR analysis revealed that the expression of miR148a was downregulated in HEK293T-IDH1R132HMUT cells as well (Fig. 3F), and that the expression level of miR148a in later passage (p23) HEK293T-IDH1R132HMUT cells could be restored by demethylation treatment with 5-aza-CdR (Fig. 3G). Interestingly, we observed downregulation of miR148a in HEK293T cells treated with 5-aza-CdR in both Fig. 3B (parental HEK293T cells) and Fig. 3G (stable HEK293T clone expressing vector only or IDH1R132H), indicating that this is a common observance with HEK293T when treated with 5 m mol/L 5-aza-CdR. Although the cause of this effect is unknown, we speculate that it may reflect direct toxicity.

To determine whether miR148a overexpression could prevent the establishment of the hypermethylator phenotype induced by mutant IDH1 (R132H), we stably overexpressed miR148a in an early passage of HEK293T stable cell lines (passage 6), which were transfected with a vector control, IDH1WT, or IDH1R132H, respectively.
The following stable cell lines were generated and cultured for an additional 20 passages: HEK293T-Control, HEK293T-IDH1WT, HEK293T-IDH1R132H, HEK293T-Control+miR148a, HEK293T-IDH1WT+miR148a, and HEK293T-IDH1R132H+miR148a. The overexpression of miR148a was monitored by GFP fluorescence and confirmed by the TaqMan real-time PCR (Supplementary Fig. S6A). By using targeted Bisq, we observed that G-CIMP genes, including RBP1 and miR148a, remained unmethylated in passage 6 of HEK293T-Control, HEK293T-IDH1WT, and HEK293T-IDH1R132H stable cell lines, which confirmed our previous finding. At passage 26, RBP1 and miR148a remained unmethylated in the HEK293T-Control and HEK293T-IDH1WT cell lines regardless of the overexpression of miR148a; however, hypermethylation occurred in the HEK293T-IDH1R132H cell line in the context of overexpression of miR148a (Supplementary Fig. S6B and S6C). Interestingly, by using real-time PCR, we were able to detect the increased expression of RBP1 in HKE293T-IDH1R132H cells when miR148a was overexpressed (p26), this suggests that partial reduction in methylation of the RBP1 CpG island may be achieved although not detectable using Bisq method (data not shown).

miR148a shows tumor-suppressive features in glioma cell lines in vitro and in vivo

To examine the biologic effects of miR148a in glioma cell lines, we overexpressed miR148a in two glioma cell lines (U251 and T98G), previously determined to have basally methylated miR148a and low miR148a expression (Fig. 3A) and compared cell growth, migration, cell cycle, and apoptosis. The efficiency of the transfection was determined by measuring the mature miRNA levels by the TaqMan qPCR (Supplementary Fig. S7) and monitored by GFP fluorescence (data not shown). The miR148a expression level relative to that of hNSC was 8- to 9-fold, raising the possibility that supra-physiologic miR148a levels were achieved. We found that overexpression of miR148a inhibited the anchorage-independent growth in soft agar (Fig. 4A), colony formation ability (Fig. 4B), and cell proliferation (Fig. 4C) of U251 and T98G glioma cells as compared with control cells. Using FACS analysis, we found that overexpression of miR148a resulted in significant G0–G1 cell-cycle arrest and a distinct decrease of S-phase cells in U251 and T98G cells as compared with control cells (Fig. 4D and Supplementary Fig. S8), and we also observed 2.8-fold (P < 0.05) increase in apoptosis in miR148a overexpressed U251 cells (Fig. 4D). Using a wound-healing assay, we measured the migration capability of the miR148a-transfected U251 cells compared with control cells and found that overexpression of miR148a inhibited cell migration (Fig. 4E).

To confirm these tumor-suppressive properties of miR148a in vivo, we tested whether miR148a could reduce tumorigenicity in both subcutaneous and intracranial glioma xenograft models. When U251 cells stably overexpressing the control EV (U251-EV control cells) or pMIF-GFP-zeo-miR148a vector (U251-miR148a cells) were injected s.c. into the flank of NOD/SCID mice, we found that the miR148a-overexpressing cell line showed much less tumor growth than that seen with control cells (Fig. 4F and G). When animals were sacrificed at 30 days after injection, we found that the tumors derived from the U251-miR148a cells were significantly smaller than those derived from U251-EV control cells (Fig. 4H). To confirm these results in an intracranial xenograft model with OS as the primary endpoint, we injected U251-EV control cells or U251-miR148a cells into the brains of NOD/SCID mice. Using the Kaplan–Meier analysis, we found that the median OS of mice injected with U251-miR148a cells (45.5 days) was greater than those of mice injected with U251-EV control cells (22.5 days; log-rank, P = 0.0005; Fig. 4I).

miR148a directly suppresses DNMT1 expression in glioma resulting in reexpression of silenced G-CIMP genes

To investigate miR148a target genes that could mediate observed tumor suppression, we identified candidate target genes by combining four well-recognized miRNA databases including TargetScan (www.targetscan.org; ref. 30), microT-CDS (DianaTools/index.php?r=mirotv4/index.), miRNA (www.microrna.org), and miRDB (www.mirdb.org), and refined the candidate gene list by annotating these genes with the TCGA gene expression data (http://tcga-data.nci.nih.gov/tcga/) analyzed for mean expression difference between IDH1MUT and IDH1WT tumors. We found 16 genes present on all four lists that were downregulated in IDH1MUT gliomas based on analysis of the TCGA gene expression database; from these genes, we selected DNMT1 for additional study (Supplementary Table S9). Consistent with this finding, it has been shown that miR148a was involved in the regulation of DNA methylation by targeting DNMT1 in breast cancer (31), gastric cancer (32), and lupus CD4+ T cells (33). Therefore, to confirm the relationship between DNMT1 expression and miR148a level in glioma, we examined the expression level of DNMT1 in glioma patient tissue samples. We confirmed that the expression of DNMT1 was slightly upregulated in IDH1MUT tissue samples (Fig. 5A and B), suggesting that there are miR148a independent regulators of DNMT1 expression. No apparent differences in DNMT1 level were observed among IDH1MUT samples separated by grade (data not shown). To determine whether miR148a downregulates the expression of DNMT1 in glioma cells, we transiently transfected U251 glioma cells with miR148a mimics or negative control mimics, and measured the mRNA and protein levels of DNMT1 by quantitative real-time PCR and Western blot analysis, respectively. Our quantitative RT-PCR results showed that in U251 cells, transfection of miR148a mimics resulted in 47% (P < 0.01) reduction of endogenous DNMT1 mRNA level after 48 hours (Fig. 5C). Western blot analysis confirmed that transfection of miR148a mimics resulted in a reduction of DNMT1 protein expression after 72 hours (Fig. 5D). In addition, downregulation of DNMT1 expression by miR148a was also observed in the glioma cells (U251 and T98G) stably overexpressing miR148a (Fig. 5E and F). By searching the TargetScan database (http://www.targetscan.org/тцgmiR148a in IDH1MUT Gliomas

CpG Island Hypermethylation of miR148a in IDH1MUT Gliomas

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org), we found that the 3′-UTR of DNMT1 contained one conserved site for miR148a (34). To determine whether this region was required for miR148a regulation, we cloned the DNMT1 3′-UTR (WT and MUT) into the pIS0 reporter vector and performed the Dual-Luciferase Assays in U251 cells, and observed a reduction of the DNMT1 3′-UTR-WT but not the MUT reporter gene expression when treated with miR148a mimics (50 nmol/L; Fig. 5G). This result was consistent with a previous study conducted in Hepa cells (35).

We next investigated the functional consequence of miR148a-regulated DNMT1 expression in terms of maintenance of hypermethylation and downregulation of other potentially important G-CIMP component genes. To do so, we selected and confirmed that representative G-CIMP genes (RBP1, DLC1, and CIDEB) with potential involvement in tumorigenesis were downregulated in IDH1MUT gliomas (Fig. 3B; and Supplementary Fig. S9; ref. 6). Upon overexpression of miR148a in U251 and T98G cells, we found that the mRNA levels of RBP1, DLC1, and CIDEB...
were upregulated compared with control cells (Fig. 5H and I). In addition, the expression of RBP1, CIDEB, and DLC1 in U251 and T98G cells could also be restored by demethyl- ation treatment with 5-aza-CdR (Fig. 5J and K). These results suggest that miR148a can diminish G-CIMP hypermethyla-
tion via DNMT1 downregulation.

Discussion

In this study, we expanded our previous RRBS character-
ization of G-CIMP (6) to examine CpG islands associated with miRNAs. We identified seven hypermethylated CpG islands at least partially within 500 base pairs of eight pre-
mirRNAs regions. This study represents the first recognition of miRNAs as part of G-CIMP and the first functional validation of tumor-suppressive effects of a G-CIMP gene, miR148a, in glioma.

We used the next-generation sequencing technique, RRBS, to perform whole methylome profiling of IDH1MUT and IDH1WT gliomas. By annotating our CpG island sequence data with the latest miRNA database, we were able to identify hypermethylated miRNAs in IDH1MUT gliomas showing that G-CIMP is composed of miRNAs in addition to coding genes. This observation was made possible by the adaptability of the RRBS dataset to annotation with previously unrecognized features. Another advantage of RRBS is conferred by the evaluation of contiguous CpG site coverage within CpG islands instead of sampling single CpG sites. However, because RRBS coverage relies on the location of naturally occurring restriction sites, we were able to cover approximately 85% of all CpG islands associated with miRNAs; in addition, our annotation does not account for the possibility that remotely located CpG islands can regulate miRNAs. Our list of hypermethylated miRNAs contains miRNAs previously found to be hyper-
methylated in glioma or other tumors. For example, miR137 has been shown to be downregulated in high-grade glioma possibly through DNA hypermethylation (36); miR34b has been shown to be silenced by promoter hyper-
methylation in multiple myeloma (37); and hypermethyla-
tion and/or downregulation of miR148a has been observed in multiple cancer types including gastric, pancreatic, lung, breast, and colorectal cancer, but until now has not been reported in gliomas (31, 32, 38–40).

To screen for functionally important hypermethylated/downregulated miRNAs, we integrated gene expression data from the TCGA GBM dataset or from our own targeted expression analysis in our tissue samples. We required that functionally important miRNAs show reduced mean expression in IDH1MUT versus IDH1WT GBMs. In doing so, we found that besides miR148a, none of the other four hypermethylated miRNAs with expression data demon-
strated correlation with decreased expression. We did not perform expression analysis on the remaining three
miRNAs. That miR148a is the only one of five hypermethylated miRNAs demonstrating reduced expression in our screen led us to explore its functional significance. Other studies have shown that hypermethylated miRNAs are often not associated with reduced expression (41, 42). Besides the obvious explanation that a hypermethylated CpG island is not involved in transcriptional regulation of the associated miRNA, another explanation is that the expression of miRNAs is too low to be detected or correlated with methylation data as seen with miR3131 in this study. Alternatively, there may be compensatory restoration of miRNAs after hypermethylation. Finally, correlation of methylation and expression pairwise in each sample, which was not possible in our study, may enable increased sensitivity of the expression filter, particularly in candidates that may be hypermethylated in only a subset of IDH1MUT samples. Therefore, the use of our "expression" filter may eliminate consideration of hypermethylated miRNA that may have functional importance. Perhaps more importantly, each hypermethylated CpG island could retain prognostic or predictive biomarker value, independent of changes in expression.

Our results show that miR148a possesses tumor-suppressive properties and is epigenetically regulated in glioma. The CpG island hypermethylation of miR148a and reduced expression has been reported in other types of cancers including lymph node metastatic cancer (40), gastric cancer (32), colon cancer (39), and breast cancer (31). This convergence of various cancer types demonstrating epigenetic miR148a silencing indicates the likely importance of miR148a dysregulation as a commonly used tumor mechanism. To address the lack of detailed understanding of the transcriptional regulation of miR148a, we demonstrated the promoter activity of a portion of the miR148a CpG island and confirmed the strong inverse correlation between promoter CpG island hypermethylation and gene expression in glioma. However, determination of the DNA hydroxymethylation levels, the accessibility of miR148a promoter to transcription factors, and the methylation/acetylation level of miR148a promoter-associated histones will be required to fully understand the mechanism of miR148a inactivation in IDH1MUT gliomas.

We show that miR148a methylation is prognostic of better outcome in newly diagnosed GBMs receiving standard chemoradiation. In contrast, it has been suggested in advanced colorectal cancer that miR148a methylation status correlated inversely with its expression, and was associated with poor OS in stage IV colorectal cancer (39). From our results, it does not appear that miR148a methylation provides independent prognostication above IDH1/2 testing. In particular, there appears to be a "false" positive rate in which hypermethylation is detected in approximately 5% patients with IDH1/2WT glioma. Although small in number, these patients do not share improved survival seen in IDH1/2MUT patients. Further study is necessary to determine the correlation of methylation with expression in this subset, and it remains to be seen whether miR148a expression level, itself, is prognostic either in IDH1/2WT or IDH1/2MUT gliomas.

Our experiments in glioma cells (U251 and T98G) clearly demonstrate the tumor-suppressive features of overexpressed miR148a in vitro and in vivo. These results are consistent with previous reports documenting tumor-suppressor features of miR148a in other cancer types (40). We used U251 and T98G cells after determining that all established GBM cell lines tested demonstrated miR148a hypermethylation and downregulated expression despite absence of IDH1/2 mutation. These results, along with the finding of a small percentage of IDH1/2WT patient tumor samples that are miR148a-methylated, indicate that miR148a methylation can occur in the absence of IDH1/2 mutation and may be important for the growth advantages necessary for these tumors or cell lines. Nonetheless, the use of non-IDH1/2MUT glioma cell lines may limit our interpretation of miR148a’s tumor-suppressive function.

To identify miR148a target genes involved in tumor-suppressive effects, we took advantage of publicly available computational-based miRNA target prediction tools and the TCGA gene expression data, looking for target candidates whose expression was significantly upregulated in IDH1MUT glioma. We identified several miR148a targets including E2F3 and ROCK1, with oncogenic features in other cancer types (40, 43). In this study, we selected DNA (cytosine-5)-methyltransferase 1 (DNMT1) for further investigation. DNMT1 is an enzyme considered to be the key maintenance CpG methyltransferase in mammals (44, 45). In other cancer types, miR148a was silenced by hypermethylation and was found to interact with DNMT1 in gastric cancer and breast cancer (31, 32). In our study, we found that the expression of DNMT1 was increased in IDH1MUT glioma and inversely correlated with miR148a expression level. We found that the overexpression of miR148a directly downregulated DNMT1 expression by targeting its 3’-UTR and restored the expression of hypermethylated G-CIMP genes in glioma cells. Further experiments involving tandem overexpression of miR148a and DNMT1 will be important to confirm the importance of this relationship. Our findings suggest that miR148a is not only regulated by DNA methylation, but itself might be involved in the maintenance of G-CIMP by targeting DNMT1. Similarly, miR29b was found to be involved in the regulation of DNA methylation by targeting DNA methyltransferase machinery in multiple diseases including acute myelogenous leukemia (AML), lung cancer, and lupus (46).

To determine whether miR148a overexpression could prevent the establishment of hypermethylator phenotype induced by mutant IDH1 (R132H), we stably overexpressed miR148a in early passage of HEK293T stable cell lines that had been transfected with vector control, IDH1WT or IDH1R132H, respectively, and monitored methylation after 20 passages. On the basis of this one time point, it appears that the hypermethylation induced by IDH1-mutant protein is not blocked by overexpression of miR148a. It is also possible that the kinetics of hypermethylation may be altered that could be detected by monitoring development of hypermethylation over several time points. Nevertheless, these results are consistent with studies in which blockade
of 2-HG generation by the mutant IDH1 enzyme could not reverse the G-CIMP (47) Overall, these results suggest that methylation of miR148a is part of G-CIMP and not the cause, although its role in maintenance of G-CIMP remains unclear.

Further study is required to determine effects of miR148a overexpression on global methylation profiles and whether G-CIMP genes preferentially undergo hypomethylation. In addition, the possibility remains whether miR148a expression may have some impact on TET demethylase activity, whose inhibition by the IDH1/2-mutant product, 2-HG, is widely hypothesized to contribute to G-CIMP hypermethylation (48, 49). However, by searching miRNA target gene databases, none of the TET family (TET1, TET2, and TET3) genes were found to be targets of miR148a or any of the miRNA candidates on Table 1 or Supplementary Table S4. We also examined the expression level of TET family genes in miR148a transient transfected U251 and T98G cells, and found that the expression level of TET1, TET2, and TET3 were nearly undetectable at baseline and transient transfection of miR148a mimic did not alter TET family gene expression level (data not shown). This suggests that miR148a might not be a direct factor that regulates TET family.

In this study, we identify miR148a as a tumor-suppressive miRNA component within G-CIMP. The ability of miR148a to have similar tumor-suppressive function across several cancer types indicates that miR148a silencing may be a broadly used mechanism. On the basis of our findings, we propose the following model for tumor promotion of IDH1-mutant gliomas (Supplementary Fig. S10) in which IDH1-mutant protein promotes a hypermethylation phenotype that includes hypermethylation and silencing of miR148a. In the poorer prognosis IDH1/2 WT gliomas in which miR148a expression is not lowered, other potumorigenic pathways are likely to be activated. Silenced miR148a results in upregulated DNMT1 levels, ordinarily suppressed by miR148a, which contributes to maintenance of hypermethylated tumor-suppressive genes within G-CIMP. Thus, reinvitation of miR148a, in this DNMT1-dependent manner, can lead to reexpression of tumor suppressors. As evident from the miR148a target list, there could be direct oncogenic targets that have increased expression in the context of silenced miR148a. Further experiments are necessary to substantiate this potential mechanism and its relative importance compared with downregulation of tumor suppressors via maintenance of G-CIMP. More importantly, further experiments are necessary to determine whether silencing of miR148a itself can be transforming or tumor promoting. Ideally, better IDH1/2-mutant cellular model systems will be available to confirm our model. Our results provide a pharmacologic rationale for the potential use of synthetic miR148a mimics as an approach for the treatment of IDH1MUT gliomas. In addition, as demonstrated by glioma cell lines and rare IDH1WT gliomas, there may be IDH1/2MUT independent mechanisms of miR148a methylation that may also be benefitted by miR148a restoration. Together with recent articles demonstrating this potential in IDH1MUT gliomas (46, 50), our results suggest possible therapeutic benefit of pharmacologic DNMT1 inhibition by FDA-approved agents to achieve miR148a reexpression in low miR148a-expressing glioma.

Disclosure of Potential Conflicts of Interest
A. Lai is a consultant/advisory board member for Genentech/Roche. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: S. Li, A.P. Chou, A. Lai
Development of methodology: S. Li, R. Chowdhury, F. Liu, A.P. Chou, T. Li, W.H. Yong, A. Lai
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.P. Chou, H. Soto, R. Prins, L.M. Liu, P.L. Nghiemphu, W.H. Yong, T.F. Cloughesy, A. Lai
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Li, R. Chowdhury, A.P. Chou, R.R. Mody, R. Prins, P.S. Mishel, P.L. Nghiemphu, T.F. Cloughesy, A. Lai
Writing, review, and/or revision of the manuscript: S. Li, R. Chowdhury, A.P. Chou, R. Prins, L.M. Liu, P.S. Mishel, W.H. Yong, T.F. Cloughesy, A. Lai
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Li, R. Chowdhury, T. Li, J.J. Lou, W. Chen, J. Reiss, T.F. Cloughesy, A. Lai
Study supervision: S. Li, A.P. Chou, H. Soto, A. Lai

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Tumor-Suppressive miR148a Is Silenced by CpG Island Hypermethylation in *IDH1*-Mutant Gliomas

Sichen Li, Reshmi Chowdhury, Fei Liu, et al.

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