

MicroRNA-142-3p is involved in regulation of *MGMT* expression in glioblastoma cells

Yi-Yen Lee,^{1,2,*} Aliksandr A Yarmishyn,^{3,4,*} Mong-Lien Wang,^{3,4} Hsiao-Yun Chen,^{4,5} Shih-Hwa Chiou,³⁻⁵ Yi-Ping Yang,^{4,5} Chun-Fu Lin,^{1,2} Pin-I Huang,^{2,6} Yi-Wei Chen,^{2,6} Hsin-I Ma,⁷ Ming-Teh Chen^{1,2}

¹Department of Neurosurgery, Neurological Institute, Taipei Veterans General Hospital, ²Faculty of Medicine, National Yang-Ming University, ³Institute of Pharmacology, National Yang-Ming University, ⁴Department of Medical Research, Taipei Veterans General Hospital, ⁵Institute of Clinical Medicine, National Yang Ming University, ⁶Cancer Center, Radiation Oncology Division, Taipei Veterans General Hospital, ⁷Department of Neurological Surgery, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan

*These authors contributed equally to this work

Correspondence: Hsin-I Ma; Ming-Teh Chen
Department of Neurological Surgery, Tri-Service General Hospital, National Defense Medical Center, 325 Section 2 Chenggong Road, Neihu, Taipei 114, Taiwan; Department of Neurosurgery, Neurological Institute, Taipei Veterans General Hospital, 201 Section 2 Shipai Road, Beitou, Taipei 112, Taiwan
Tel +886 2 8792 7191; +886 2 2875 7491 ext 9
Email uf004693@mail2000.com.tw; mtchen@vghtpe.gov.tw

Background: Glioblastoma multiforme (GBM) is the most malignant brain tumor, and there is no effective treatment strategy. Patients with GBM have a median overall survival of only 14.6 months. Current treatment consists of safe and maximal surgical excision, followed by concurrent chemoradiotherapy and maintenance chemotherapy. There are several obstacles that hinder the effectiveness of this aggressive treatment. Temozolomide (TMZ) is an oral alkylating drug that acts through alkylating the *O*⁶ position of guanine in DNA that leads to cell death. However, the expression and enzymatic activity of the DNA repair protein *MGMT* limits the therapeutic benefit from treatment with TMZ. *MGMT* reduces the efficacy of alkylating drugs by removing the methyl or alkyl group from damaged *O*⁶-methylguanine. Expression levels of *MGMT* play an important role in the outcome of GBM patients. miRNAs are a group of small regulatory RNAs that control target gene expression by binding to mRNAs. miR-142-3p has been found to be an important factor in the development and maintenance of the oncogenic state.

Results: In this study, we sought to investigate whether miR-142-3p can regulate *MGMT* gene expression in GBM cells. Here, we show that miR-142-3p downregulates *MGMT* expression through binding to the 3'-UTR of *MGMT* mRNA, thus affecting protein translation. Responsiveness to TMZ was significantly enhanced after transfection with miR-142-3p. Overexpression of miR-142-3p also sensitized GBM cells to alkylating drugs.

Conclusion: Above all, our findings demonstrate that miR-142-3p plays a critical role in regulating *MGMT* expression, has great potential for future clinical applications, and acts as a new diagnostic marker for this intractable disease.

Keywords: glioblastoma, *O*⁶-methylguanine-DNA methyltransferase, miR-142-3p, carmustine, temozolomide

Introduction

Glioma is a common type of brain tumor originating from glial cells that support and nourish the brain neurons. Glioblastoma multiforme (GBM) is the most aggressive type of glioma and is classified by the World Health Organization as a grade IV tumor. GBM accounts for >50% of all gliomas.¹ GBM is characterized by pleomorphism, rapid proliferation, high invasiveness, and the capability to infiltrate and destroy the surrounding areas of the brain. Angiogenesis also occurs in neighboring tissues, which further enhances tumor growth and invasion.²⁻⁴ The estimated median survival of GBM patients is 14.6 months after diagnosis.⁵ Surgery cannot prevent recurrence, because tumor cells that spread and infiltrate into the surrounding brain tissue cannot be completely removed. Furthermore, the effectiveness of chemotherapeutic drugs is largely

limited by the blood–brain barrier (BBB), which excludes most chemotherapeutic drugs from entering the brain.⁶

Most chemotherapeutic drugs for GBM treatment are alkylating agents that cross the BBB, such as carmustine (bis-chloroethylnitrosourea [BCNU]) and temozolomide (TMZ). Alkylating agents methylate guanine on the oxygen atom, thus forming *O*⁶-methylguanine residues in the nuclear DNA. *O*⁶-methylguanine forms cytotoxic DNA cross-links, which lead to consequent apoptosis of tumor cells.⁷ TMZ undergoes rapid chemical conversion in the systemic circulation under physiological conditions to become the active compound 5-(3-methyltriazene-1-yl)-imidazole-4-carboxamide (MTIC). The cytotoxicity of MTIC is primarily due to alkylation of DNA, mainly occurring at the *O*⁶ and *N*⁷ positions of guanine residues.⁸ Currently, concomitant and adjuvant TMZ is applied with radiation in order to improve the survival of GBM patients.^{1,5} However, MGMT limits the therapeutic benefit from treatment with TMZ.^{5,7,9,10} Hypermethylation of the *MGMT* promoter is associated with improved survival of GBM patients.¹¹ The *MGMT* gene is located on chromosome 10q26 and consists of five exons. MGMT is a DNA repair protein that removes cytotoxic *O*⁶-methylguanine or *O*⁴-alkyl-thiamine lesions in DNA induced by drugs or exogenous agents. After receiving and covalently binding methyl/alkyl groups, MGMT is irreversibly inactivated and degraded through the ubiquitination-dependent pathway.¹²

Recently, it has become increasingly clear that miRNAs can function as either tumor suppressors or oncogenes.¹³ The dysregulation of miRNA expression has been implicated in the formation of specific cancers. Most miRNAs decrease the expression of tumor suppressors, leading to increased proliferation, invasion, and angiogenesis, and decreased cell death, thus resulting in the development of tumors. The underlying mechanisms include the activation of promoters, elevated biosynthesis, and enhanced stability of miRNAs.^{14–17} miR-142-3p is generated and processed from the *MIR142* gene, located on the human chromosome 17q22. Several studies indicate that miR-142-3p can be involved in cancers. For example, it has been shown that miR-142-3p can inhibit the expression of the *RAC1* gene in hepatocellular carcinoma cell lines, which results in suppression of their migration and invasion capacity.¹⁸ Similarly, miR-142-3p is able to inhibit proliferation and invasion of cervical cancer cells by targeting *FZD7*.¹⁹ In non-small-cell lung carcinoma cells, miR-142-3p can repress TGF β -induced growth inhibition by targeting TGF β R1, thus acting as an oncogene in this type of cancer.²⁰ Research on GBM cells has shown that IL6 inhibits miR-142-3p expression via increased methylation of its promoter. At the same time, IL6 expression is also suppressed by miR-142-3p, thus

forming negative-feedback loop-based interplay between these two factors.²¹ It has previously been demonstrated that acquisition of TMZ resistance in GBM cells can be controlled by miRNAs, such as miR-195, miR-445-3p, and miR-10a.²²

Therefore, in this study, we aimed to investigate whether miR-142-3p can regulate *MGMT* gene expression and to explore the effect of this regulation on resistance to alkylating agents in GBM cells. We demonstrate that miR-142-3p and MGMT had negatively correlated the patterns of expression in GBM cell lines. Furthermore, we show that miR-142-3p suppresses translation of *MGMT* in a 3'-UTR-dependent manner, but does not have any effect on *MGMT* mRNA levels. Importantly, overexpression of miR-142-3p sensitized GBM cells to the alkylating drugs BCNU and TMZ.

Materials and methods

Cell culture

GBM cell lines were acquired from the Neurological Institute of Veterans General Hospital under the supervision and approval of the Taipei Veterans General Hospital institutional review board. The GBM cell lines S1 and R1 were maintained in MEM (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% FBS, nonessential amino acids, sodium pyruvate, L-glutamine, and 1% penicillin–streptomycin at 37°C and 5% CO₂. HEK293T/17 cells were cultured in DMEM containing 10% FBS (Thermo Fisher Scientific) and 1% penicillin–streptomycin.

Vector constructs

For overexpression of miR-142-3p, its precursor sequence was cloned into a pLKO.1-puro vector (Addgene, Cambridge, MA, USA). Lentiviral particles were produced by cotransfecting HEK293T cells (ATCC, Manassas, VA, USA) with pLKO.1-puro, pCMV-dR8.91 (Addgene), and pCMV-VSV-G (Addgene) using standard calcium phosphate transfection. The virus was harvested at 48 hours posttransfection and purified by filtering culture supernatant through a 0.45 μ m filter. S1 and R1 cells were infected with lentiviral particles by incubation for 24 hours in the presence of 8 μ g/mL polybrene.

For luciferase reporter assays, full-length *MGMT* 3'-UTR (1–522 nucleotides) and its truncated forms 3'-UTR-del1 (1–437 nucleotides), 3'-UTR-del2 (1–229 nucleotides), and 3'-UTR-del3 (1–127 nucleotides) were amplified by polymerase chain reaction (PCR) from cDNA using the SpeI-tagged forward primer GCACTAGTGTATGTGCAGTAGGATG and the HindIII-tagged reverse primer AGAAGCTTCTCTGTAAGCACTGACT to amplify full-length 3'-UTR, ATAAGCTTATGCCATCACAGCCATGGACACAGC

to amplify 3'-UTR-del1, ATAAGCTTAATGGACAT-GAAAAATAGAGCAAGG to amplify 3'-UTR-del2, and ATAAGCTTAGAAAGGGCAGACACGCTTGTTCCC to amplify 3'-UTR-del3. PCR products were ligated into SpeI-HindIII restriction sites of pMIR-REPORT Luciferase vector (Thermo Fisher Scientific). The resultant constructs were transfected into HEK293T cells using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions.

Reverse-transcriptase PCR and quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Total RNA (5 µg) was transcribed into cDNA using SuperScript III reverse transcriptase (RT; Thermo Fisher Scientific) according to the manufacturer's instructions. The following pairs of primers were used to amplify the respective transcripts: *MGMT* (forward GCCCCTAGAACGCTTTGCGTCCCGA, reverse CCCTGCTCACAACCAGACAGCTCCA), miR-142-3p (forward TGCAGGGCAGCAGAGGAGCTGCTGT, reverse ACTGAGGCTCTGGGCAGTCAGGACC), and *GAPDH* (forward: CTCATGACCACACTCCATGC, reverse: TTCAGCTCTGGGATGACCTT). For RT-PCR, Takara Taq polymerase was used and reaction products resolved by agarose gel electrophoresis. For quantitative real-time (qRT)-PCR, SYBR green PCR Master Mix was used and reactions run on an Applied Biosystems 7900HT fast real-time PCR system (Thermo Fisher Scientific).

Methylation-specific PCR

Genomic DNA was extracted from cell lysates using the standard phenol:chloroform:isoamyl alcohol extraction method. Genomic DNA was treated with bisulfite prior to performing PCR. Two 35-cycle rounds of PCR were performed: during the first PCR, *MGMT* promoter methylated-specific (forward TTTCGACGTTTCGTAG-GTTTTGCGC, reverse GCACTCTTCCGAAAACGAAACG) and unmethylated-specific (forward TTTGTCTTTTGAT-GTTTGTAGGTTTTTGT, reverse AACTCCACACTCTTCCAAAACAAAACA) primer pairs were used. During the second PCR, nested primers (forward GYGTTTYGGATAT-GTTGGGATAGTT, reverse AACTCCRCACACTCTTCCRAAAAC) were used to amplify the 10× diluted first PCR amplification product. PCR products were resolved in 2%–3% agarose gel and visualized under ultraviolet light after staining with ethidium bromide.

Luciferase assay

HEK293T cells grown in 12-well dishes (5×10^4 cells/well) were transfected with the appropriate pMIR-REPORT Luciferase constructs and pMIR-REPORT β-galactosidase reporter control vector for normalizing transfection efficiency, using the standard Lipofectamine 2000 protocol. Luciferase assays were performed at 48 hours posttransfection using a Dual-Light system (Thermo Fisher Scientific) according to the manufacturer's protocol. Briefly, cells were lysed in lysis solution containing 0.5 mM dithiothreitol and transferred into microcentrifuge tubes. The lysate was centrifuged at 13,200 rpm at 4°C for 2 minutes to remove cellular debris. Lysate (5 µL) was transferred into an assay cuvette, to which 12.5 µL buffer A and 50 µL buffer B (containing 100× diluted Galacton-Plus substrate) were added. Chemiluminescence detection of luciferase activity was performed using a luminometer. Samples were maintained at room temperature for 30–60 minutes. The activities of β-galactosidase in samples were determined by the addition of a Light Emission Accelerator-II.

Cell viability assay

S1 and R1 cells ($1-2 \times 10^4$ cells/mL) were seeded into 24-well plates and incubated overnight. On the next day, fresh media with different concentrations of drugs (0, 15, 30, 60, 125, and 250 µM BCNU (Sigma-Aldrich, St Louis, MO, USA) and 0, 15, 30, 60, 125, 250, 500, and 1,000 µM TMZ (Sigma-Aldrich) were added to the wells. Each treatment was performed in triplicate. After 48 hours of incubation with drugs, aliquots of fresh media containing 500 µg/mL MTT (Sigma-Aldrich) were added to each well, followed by incubation at 37°C for 1–2 hours in the dark. Medium (200 µL) was transferred into 96-well plates and absorbance measured at 560 and 670 nm using an enzyme-linked immunosorbent assay reader. IC₅₀ was determined by Excel and graphs were plotted using SigmaPlot.

Results

Expression profiles of MGMT protein and miR-142-3p in different GBM cell lines

Initially, we sought to investigate the expression patterns of MGMT protein and miR-142-3p in different GBM cell lines. We used in-house S1 and R1 cell lines derived from patients with first-diagnosed GBM and recurrent GBM, respectively, as well as popular commercially available GBM cell lines: LN18, U87MG, GBM8401, and GBM8901. The control fetal glial cell line SVG p12 was used as a representative of normal glial cells. Using Western blotting, we observed high levels

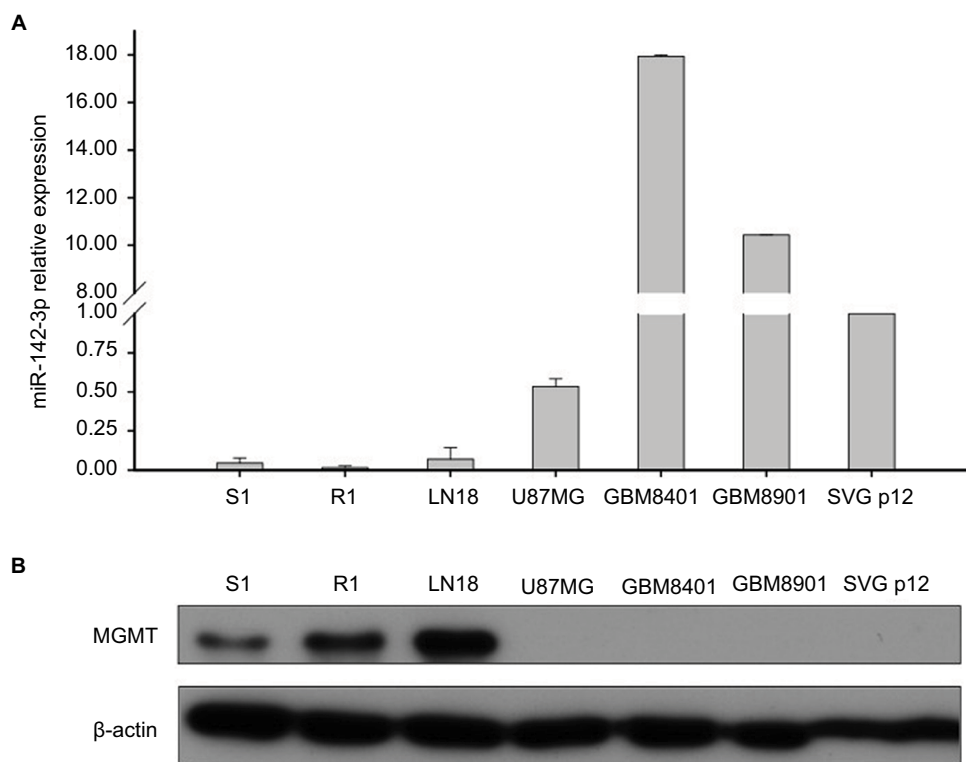


Figure 1 Expression of MGMT and miR-142-3p in GBM cell lines.

Notes: (A) qRT-PCR analysis of expression of miR-142-3p in GBM cell lines S1, R1, LN18, U87MG, GBM8401, GBM8901, and fetal glial SVG p12 cells. Expression levels in GBM cell lines quantified relative to expression levels in the SVG p12 cell line. (B) Western blotting analysis of MGMT expression in GBM cell lines. β -Actin was detected as a loading control.

Abbreviations: GBM, glioblastoma multiforme; qRT-PCR quantitative real-time polymerase chain reaction.

of MGMT protein expression in GBM cell lines S1, R1, and LN18; however, its expression was not detected in U87MG, GBM8410, GBM8901, or SVG p12 cells (Figure 1B). In contrast, using qRT-PCR, we found that miR-142-3p was expressed at lower levels in the cell lines with higher levels of MGMT protein expression (Figure 1A). Based on these findings, further miR-142-3p gain-of-function experiments were performed using the cell lines S1 and R1, which had the lowest endogenous levels of miR-142-3p and at the same time expressed high levels of MGMT protein.

Marginal effect of overexpression of miR-142-3p on MGMT promoter methylation in GBM cell lines

Preliminary results indicated that GBM cell lines with higher MGMT protein expression exhibited lower miR-142-3p expression. Many previous studies have indicated that GBM patients with a methylated MGMT promoter were more resistant to alkylating agents, and thus, their treatment efficacy was reduced. Therefore, we tested the effect of miR-142-3p

overexpression on the methylation status of the MGMT promoter. For this purpose, we used lentivirus vector to overexpress miR-142-3p in S1 (Figure 2C) and R1 cell lines (Figure 2D), using low and high multiplicities of infection. The effect of overexpressed miR-142-3p on methylation status of the endogenous MGMT promoter was analyzed using methylation-specific PCR (Figure 2A and B). Overexpression of miR-142-3p marginally enhanced methylation of the MGMT promoter compared with the control (shLuc) in both S1 (Figure 2A) and R1 (Figure 2B) cell lines.

Overexpression of miR-142-3p does not affect MGMT mRNA, but decreases MGMT protein level

As the next step, we examined whether miR-142-3p overexpression affected the expression of MGMT at an mRNA or a protein level (Figure 3). S1 and R1 cells were infected with miR-142-3p-encoding recombinant lentiviruses, and levels of MGMT mRNA were analyzed by qRT-PCR. Overexpression of miR-142-3p did not change MGMT mRNA levels in

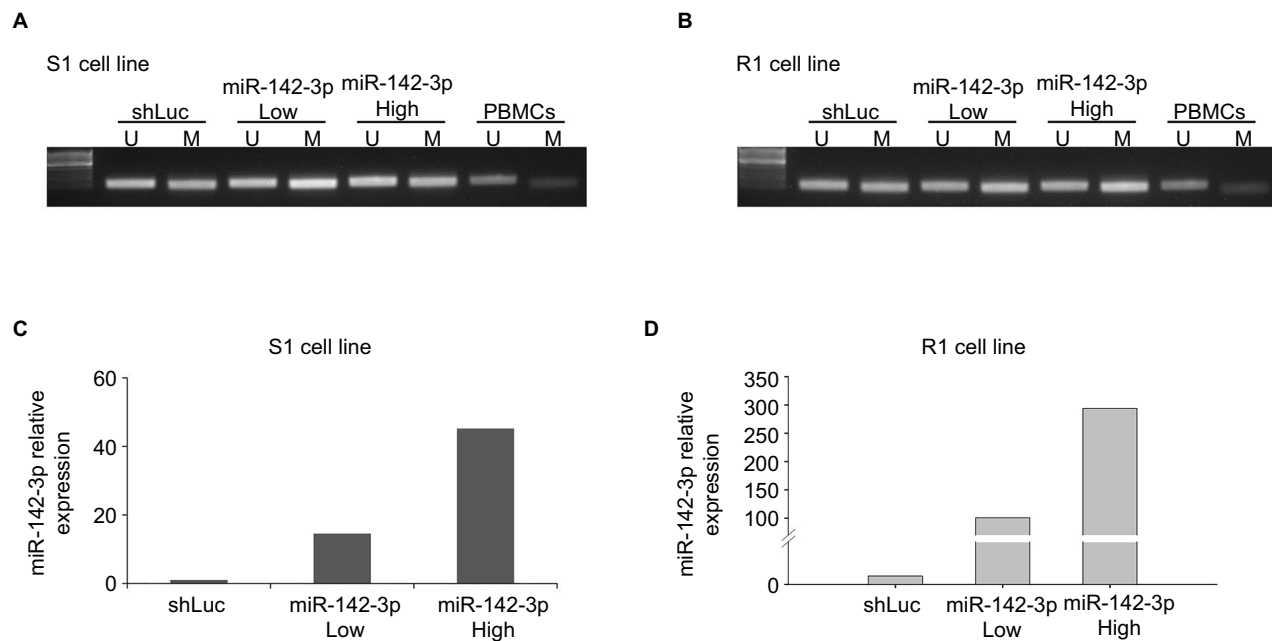


Figure 2 Overexpression of miR-142-3p increases *MGMT* promoter methylation in GBM cell lines.

Notes: Methylation-specific PCR assay showing methylation status of *MGMT* promoter in S1 cells (**A**) and R1 cells (**B**) infected with control (shLuc) and miR-142-3p-encoding recombinant lentivirus at low and high multiplicities of infection. Lane U represents amplification of unmethylated *MGMT* promoter and lane M represents methylated *MGMT* promoter. Total DNA extracted from PBMCS was used as control: lane U represents amplification of unmethylated PBMCS DNA and lane M represents amplification of methylated PBMCS DNA pretreated with *SssI* methyltransferase. qRT-PCR showing overexpression of miR-142-3p in S1 cells (**C**) and R1 cells (**D**) after infection with recombinant lentivirus at low (Low) and high (High) multiplicities of infection. Expression levels in miR-142-3p-overexpressing cells quantified relative to expression level in control cells infected with shLuc lentiviral construct, with $\Delta\Delta C_t$ quantification with *GAPDH* amplification used as internal control.

Abbreviations: GBM, glioblastoma multiforme; qRT-PCR quantitative real-time polymerase chain reaction; PBMCS, peripheral blood mononuclear cells.

either S1 (Figure 3A) or R1 (Figure 3B) cell lines. On the other hand, *MGMT* protein levels examined by Western blotting were noticeably reduced in both S1 (Figure 3C) and R1 (Figure 3D) cells, with more pronounced effect at the high multiplicity of infection.

miR-142-3p binds *MGMT* 3'-UTR directly to regulate *MGMT* gene

The aforementioned findings showed that increased expression of miR-142-3p suppressed the expression of *MGMT* protein, but *MGMT* mRNA expression was not affected. To determine whether this effect of miR-142-3p on *MGMT* expression was dependent on complementary binding to the 3'-UTR of *MGMT* mRNA, the 3'-UTR sequence of *MGMT* was cloned downstream of the luciferase-coding sequence in the luciferase-reporter vector pMIR-REPORT Luciferase. Easily transfectable HEK293T cells were cotransfected with the *MGMT* 3'-UTR-luciferase construct and different amounts (0, 50, 100, and 150 ng) of plasmid expressing miR-142-3p (pLKO-miR-142-3p). As shown in Figure 4A, miR-142-3p overexpression significantly decreased luminescence generated by *MGMT* 3'-UTR luciferase reporter in a dose-dependent manner. To confirm further that decreased luminescence in HEK293T cells was induced

by the inhibitory action of miR-142-3p on *MGMT* 3'-UTR, cells were cotransfected with 0, 15, and 30 ng of an miR-142-3p-specific miRNA sponge construct. The effects of the miRNA sponge were determined by luminescence measurement in HEK293T cells cotransfected with *MGMT* 3'-UTR and miR-142-3p. miR-142-3p diminished luminescence in HEK293T cells in the absence of the miRNA sponge; however, in cells cotransfected with 15 and 30 ng miRNA sponge, miR-142-3p-induced decrease in luminescence was not observed (Figure 4B).

As our findings implied that miR-142-3p mediated *MGMT* mRNA translation by direct interaction with its 3'-UTR, we tried to predict the binding site using the common miRNA target prediction tools TargetScan, PicTar, and miRanda. However, no specific miR-142-3p-binding site was identified, which could have been due to the design of these algorithms, which are based on 3'-UTR alignment with the region between the second and eighth nucleotides of miRNA, ignoring the possibility of base pairing with other regions. Therefore, we aimed to identify the miR-142-3p-interacting region within *MGMT* 3'-UTR experimentally. For this purpose, we constructed luciferase-reporter constructs with various truncated fragments of *MGMT* 3'-UTR, as shown in Figure 4C, and coexpressed them with different doses

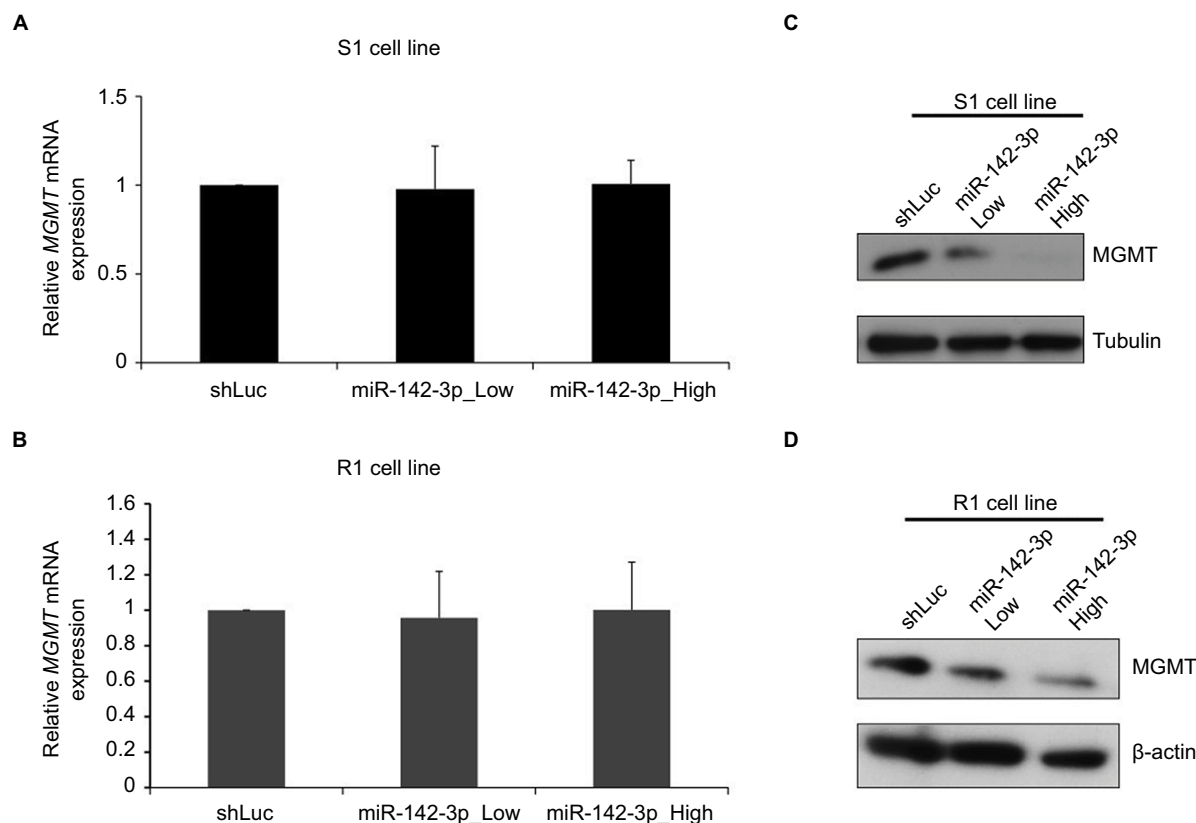


Figure 3 Overexpression of miR-142-3p does not affect *MGMT* mRNA, but decreases *MGMT* protein level.

Notes: qRT-PCR analysis of expression of *MGMT* mRNA in S1 (**A**) and R1 cells (**B**) infected with control (shLuc) and miR-142-3p-encoding recombinant lentiviruses at low and high multiplicities of infection. Expression levels of *MGMT* mRNA in miR-142-3p-infected cells quantified relative to control shLuc-infected cells and presented as mean from three independent experiments with SD error bars. Western blotting analysis of expression of *MGMT* protein in S1 (**C**) and R1 cells (**D**) after infection with shLuc control and miR-142-3p-encoding recombinant lentivirus at low and high multiplicities of infection. β -Actin and tubulin were used as loading controls.

Abbreviation: qRT-PCR quantitative real-time polymerase chain reaction.

of miR-142-3p (Figure 4D). The results showed that miR-142-3p markedly reduced only the luciferase activity of the full-length *MGMT* 3'-UTR reporter construct (3'-UTR-FL) in a dose-dependent manner, but did not affect other reporter constructs with truncated 3'-UTRs (3'-UTR-del1, 3'-UTR-del2, and 3'-UTR-del3; Figure 4D).

miR-142-3p overexpression inhibits GBM-cell viability after treatment with alkylating agents

Current treatment regimens for GBM patients include alkylating agents that cross the BBB. However, outcomes are limited by the level and activity of *MGMT* expression. Given the fact that elevated miR-142-3p can inhibit *MGMT* protein expression (Figure 2), we analyzed the cytotoxic and survival effects of increased miR-142-3p expression in GBM cell lines. Two alkylating drugs – BCNU dissolved in ethanol and TMZ dissolved in dimethyl sulfoxide – were tested. The cell viabilities were assessed by MTT assay after incubation with different concentrations of alkylating drugs

for 72 hours. As shown in Figure 5, the IC_{50} values of both BCNU and TMZ decreased in S1 and R1 cells infected with miR-142-3p-encoding recombinant lentivirus compared to the control. Moreover, this decrease in IC_{50} values was more pronounced in the case of BCNU treatment compared to TMZ treatment. As such, our findings demonstrate that increased miR-142-3p expression can reduce resistance to alkylating drugs in GBM cell lines.

Discussion

It is commonly known that miRNAs are important regulatory molecules whose dysregulation is widely implicated in oncogenesis.²³ Similarly to other miRNAs, miR-142-3p has been shown to be involved in different kinds of cancer by regulating various targets implicated in both oncogenic and tumor-suppressor pathways.²⁴ For example, in non-small-cell lung carcinoma cells, miR-142-3p can repress TGF β -induced growth inhibition by targeting the TGF β R1 receptor, thus acting as an oncogene.²⁰ On the other hand, tumor suppressor functions of miR-142-3p are often associated with targeting

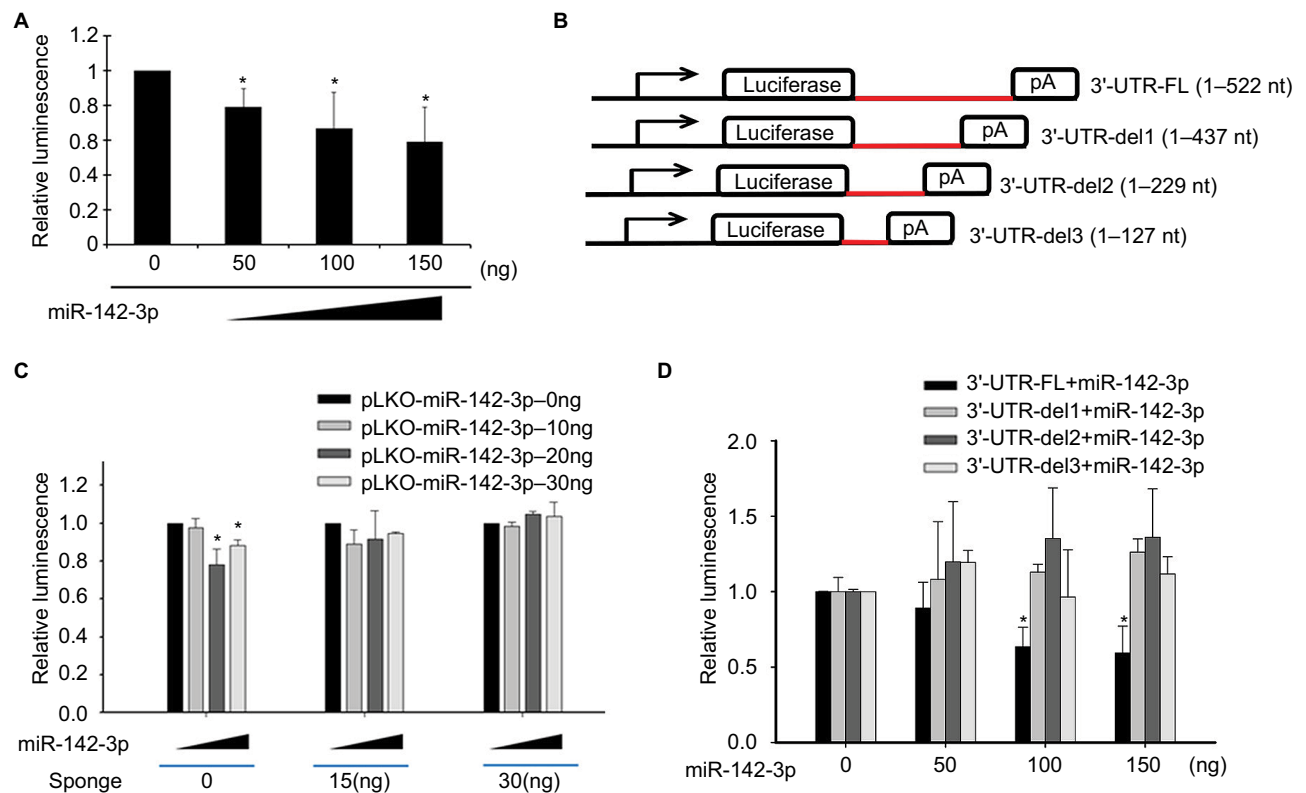


Figure 4 Regulatory effect of miR-142-3p on MGMT 3'-UTR.

Notes: (A) Luciferase-reporter assay demonstrating the effect of miR-142-3p on MGMT 3'-UTR-mediated luciferase activity. The luciferase-reporter construct (pMIR-REPORT Luciferase) containing MGMT 3'-UTR insert was cotransfected into HEK293T cells with the indicated amounts of miR-142-3p-encoding plasmid (pLKO-miR-142-3p) and luminescence determined at 48 hours posttransfection. Readouts from three wells were normalized by β -galactosidase activity and expressed as mean with SD error bars. * $P < 0.05$ (Student's *t*-test). (B) Inhibitory effect of miR-142-3p overexpression on MGMT 3'-UTR-mediated luciferase activity reduced by coexpression of miR-142-3p-specific miRNA sponge. MGMT 3'-UTR-tagged pMIR-REPORT Luciferase construct was cotransfected into HEK293T cells with different amounts (0, 50, 100, and 150 ng) of pLKO-miR-142-3p and 0, 15, and 30 ng of miRNA sponge construct. Luminescence was determined at 48 hours posttransfection. Readouts from three wells were normalized by β -galactosidase activity and expressed as mean with SD error bars. * $P < 0.05$ (Student's *t*-test). (C) Design of luciferase reporter tagged with full-length MGMT 3'-UTR (3'-UTR-FL [1–522 nucleotides]) and three truncated MGMT 3'-UTRs (3'-UTR-del1, 3'-UTR-del2, and 3'-UTR-del3). (D) miR-142-3p-dependent suppression of luciferase activity mediated by FL MGMT 3'-UTR, but not by its truncated forms. pMIR-REPORT Luciferase reporter vectors with various lengths of MGMT 3'-UTR (FL, del1, del2, and del3) were cotransfected into HEK293T cells with different amounts of pLKO-miR-142-3p (0, 50, 100, and 150 ng). After 48 hours, luciferase activity was measured and normalized by β -galactosidase activity. Experiments were performed with three replicates, with mean and SD error bars shown. * $P < 0.05$ (Student's *t*-test).

the HMG family of proteins, such as HMGB1 in lung cancer^{25,26} and osteosarcoma,²⁷ HMGA1 in osteosarcoma,²⁸ and HMGA2 in GBM.²¹ In our recent study, we found that oncogenic cytokine IL6 promotes GBM propagation by suppressing expression of miR-142-3p.²¹ Given the fact that high expression of the DNA repair protein MGMT is one of the most crucial factors of poor prognosis in GBM patients,²⁹ we sought to investigate a possible functional link between miR-142-3p and MGMT. Therefore, we tested the expression levels of miR-142-3p and MGMT protein in different GBM cell lines and found that they were negatively correlated. For our further experiments, we used two GBM cell lines, S1 and R1, derived from patients with first-diagnosed and recurrent GBM, respectively, in which miR-142-3p was expressed at very low levels and MGMT was highly expressed (Figure 1). In comparison, in several other tested GBM cell lines and the normal glial cell line SVG p12, the high level of expression

of miR-142-3p correlated with the absence of expression of MGMT (Figure 1). Such patterns of expression made S1 and R1 cells a good model for miR-142-3p gain-of-function study to investigate whether miR-142-3p has an effect on MGMT expression.

Here, we demonstrated that overexpression of miR-142-3p suppressed MGMT protein expression; however, it did not have any effect on its mRNA levels (Figure 3), which indicated that miR-142-3p might inhibit translation of MGMT mRNA, but did not affect its stability. As is commonly known, the regulatory functions of miRNAs depend on the base-pairing region between the second and seventh nucleotides of miRNA, known as the seed region, which binds the 3'-UTR of a target gene. Currently, most analyses also follow this canonical miRNA-binding rule to predict the potential targets of miRNAs. However, we were unable to predict a binding site of miR-142-3p within

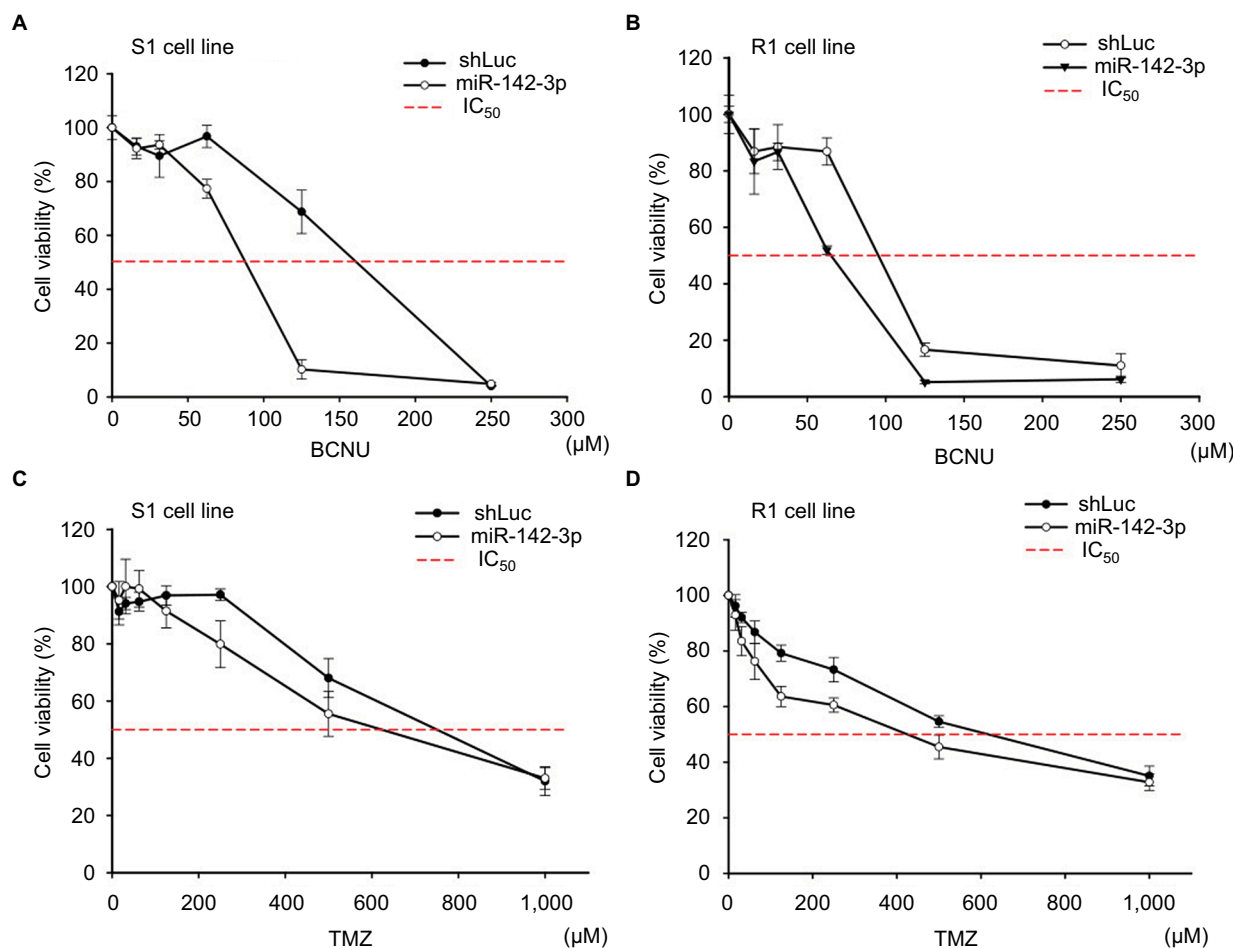


Figure 5 Overexpression of miR-142-3p leads to reduction of resistance to alkylating drugs in GBM cell lines.

Notes: S1 cells (**A, C**) and R1 cells (**B, D**) were infected with control (shLuc) and miR-142-3p-encoding recombinant lentiviruses and treated with the indicated concentrations of BCNU (**A, B**) or TMZ (**C, D**). Cell viability was determined by MTT colorimetric assay after 48 hours of incubation. Cell viability of drug-treated cells expressed as percentage of 560/670 nm absorbance values relative to untreated cells (100%). Three independent measurements were performed for each experimental condition.

Abbreviations: GBM, glioblastoma multiforme; BCNU, bis-chloroethylnitrosourea (carmustine); TMZ, temozolomide.

the *MGMT* 3'-UTR using the available bioinformatic tools (TargetScan, PicTar, and miRanda). Nevertheless, using the luciferase-reporter assay, we have experimentally proven that miR-142-3p inhibits translation of *MGMT* mRNA via a 3'-UTR-binding mechanism (Figure 4). Moreover, by testing the ability of different truncated forms of *MGMT* 3'-UTR to mediate the repressive effect of miR-142-3p on translation, we showed that only the full-length 3'-UTR consisting of 522 nucleotides was capable of this. Given that the shortest tested truncated form comprised nucleotides 1–437 of the 3'-UTR, we could assume that nucleotides 438–522 of *MGMT* 3'-UTR contained an miR-142-3p-response element. miR-142-3p has partial complementarity with this region in at least two possible ways, albeit without a particular bias to the seed region (Figure 6A). In a number of recent studies, the roles of miRNAs involved in regulating downstream gene expression were not limited

to the seed region, and binding sites were not only within the sequence of a target-gene 3'-UTR. miRNAs also show the inhibitory activities through binding to the 5'-cap region and open reading frames of target genes.^{30–33} The genome-wide mapping of miRNA–mRNA interaction sites in mouse brain revealed that a significant proportion of them were mediated by noncanonical seed-independent mechanisms.^{34,35} Based on a recently described mechanism in *Caenorhabditis elegans*, although noncanonical interaction may not be sufficient on its own to recruit the miRNA-induced silencing complex, it facilitates its recruitment by neighboring canonical seed-pairing interactions.³⁶ Interestingly, two miRNAs, miR-181d-5p and miR-409-3p, have been shown cooperatively to suppress *MGMT* expression, augmenting each other's effect.³⁷ Therefore, in spite of the apparent noncanonical mode of interaction of miR-142-3p with *MGMT* 3'-UTR, it may exert its effect by cooperating with

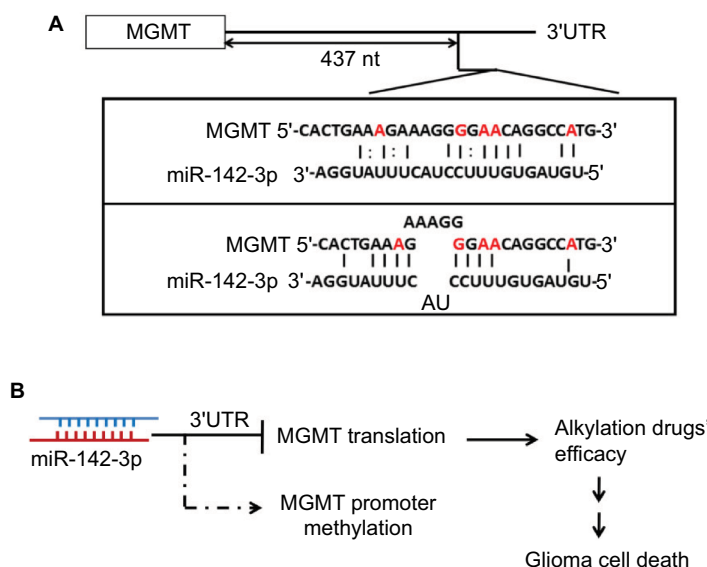


Figure 6 Model of interaction between miR-142-3p and MGMT.

Notes: (A) Best complementarity of miR-142-3p to 3'-UTR of *MGMT* mRNA within the region 438–522 nucleotides, experimentally proven to mediate the repressive effect on translation. (B) miR-142-3p inhibits *MGMT* mRNA translation and thus diminishes MGMT protein level by binding to *MGMT* 3'-UTR. Therefore, overexpression of miR-142-3p in GBM cell lines enhanced alkylating agents' efficacy, which caused GBM cell death.

Abbreviation: GBM, glioblastoma multiforme.

other miRNAs. In a recent study, it was shown that miR-603 directly regulated *MGMT* expression and targeted *MGMT* mRNA on five 3'-UTR sites.³⁸ One of these sites is located adjacently to our predicted miR-142-3p response element (Figure 6A), which makes miR-603 a good candidate for a partner of miR-142-3p in a cooperative model of miRNA-induced silencing-complex recruitment.

Many previous studies have indicated that methylation of the *MGMT* promoter is associated with treatment outcomes in GBM patients.^{39,40} In the current study, we have shown that the ratio of methylated:unmethylated *MGMT* promoter was slightly increased in GBM cell lines, exogenously overexpressing miR-142-3p, whereas the *MGMT* mRNA level was not affected. Although this effect was very mild and did not result in a change in *MGMT* mRNA levels in S1 or R1 cells, it is highly possible that it could be more pronounced in a different cellular environment of other cell lines.

Currently, the best standard treatment of GBM is maximal safe surgical resection, followed by concurrent radiochemotherapy and TMZ maintenance. However, the options of chemotherapeutic drugs are limited by drug resistance. Although alkylating agents are characterized by better BBB permeability, their efficacy is decreased by the DNA-repairing capability of MGMT protein. *MGMT* expression is known to be negatively regulated epigenetically by methylation of its promoter. Traditionally, MGMT

promoter methylation is the most commonly used predictor for alkylating agent treatment outcome.^{9,41} However, different studies have produced ambiguous results on the predictive power of *MGMT* promoter methylation, as high methylation status is not always associated with positive alkylating agent treatment outcome in GBM patients.^{42,43} In addition, this criterion is not applicable to 60% of GBM patients, who have unmethylated *MGMT* promoters, but still can be potentially responsive to alkylating agent treatment.⁴⁴ Therefore, given the fact that *MGMT* expression is abundantly regulated at the posttranscriptional level by miRNAs, it makes this class of molecules good candidates as potential biomarkers to predict alkylating agent treatment.⁴⁵ In cell viability assays, we observed that overexpression of miR-142-3p increased the resistance of S1 and R1 cells to the alkylating drugs TMZ and BCNU, commonly used in chemotherapy of GBM, which was highly consistent with the molecular effect of suppression of MGMT activity by miR-142-3p (Figure 5). We also observed that miR-142-3p overexpression was more efficient in increasing the sensitivity to BCNU compared to TMZ, and thus, it may potentially be considered to be a better prognostic factor for the former drug, a possibility that can be tested in clinical studies.

Conclusion

To summarize, in our study, we found that miR-142-3p expression was negatively correlated with MGMT protein

levels in GBM cells. Overexpression of miR-142-3p in MGMT-expressing GBM cell lines S1 and R1 diminished MGMT protein levels, but not mRNA levels. Our data indicate that miR-142-3p downregulates MGMT through binding to *MGMT* 3'-UTR by a noncanonical seed-independent mechanism. *MGMT* promoter methylation status was slightly increased after miR-142-3p overexpression; however, it did not result in changes in transcription. miR-142-3p overexpression inhibited GBM cell viability after treatment with alkylating drugs, commonly used in chemotherapy for GBM, particularly to BCNU (Figure 6B). This makes miR-142-3p a good candidate as a potential biomarker and a therapeutic target in GBM.

Acknowledgments

This study was funded by the Ministry of Science and Technology (MOST; 106-2633-B-009-001, 104-0210-01-09-02, 105-0210-01-13-01, and 106-0210-01-15-02), NHRI-EX106-10621BI, Taipei Veterans General Hospital (V104B-036), and the Department of Health Cancer Center Research of Excellence (MOHW106-TDU-B-211-113001 and MOHW106-TDU-B-211-144003).

Disclosure

The authors report no conflicts of interest in this work.

References

- Friedman HS, Kerby T, Calvert H. Temozolomide and treatment of malignant glioma. *Clin Cancer Res*. 2000;6(7):2585–2597.
- Birnbaum T, Hildebrandt J, Nuebling G, Sostak P, Straube A. Glioblastoma-dependent differentiation and angiogenic potential of human mesenchymal stem cells in vitro. *J Neurooncol*. 2011;105(1):57–65.
- di Tomaso E, Snuderl M, Kamoun WS, et al. Glioblastoma recurrence after cediranib therapy in patients: lack of “rebound” revascularization as mode of escape. *Cancer Res*. 2011;71(1):19–28.
- Smits M, Nilsson J, Mir SE, et al. miR-101 is down-regulated in glioblastoma resulting in EZH2-induced proliferation, migration, and angiogenesis. *Oncotarget*. 2010;1(8):710–720.
- Stupp R, Mason WP, van den Bent MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med*. 2005;352(10):987–996.
- Agarwal S, Sane R, Oberoi R, Ohlfest JR, Elmquist WF. Delivery of molecularly targeted therapy to malignant glioma, a disease of the whole brain. *Expert Rev Mol Med*. 2011;13:e17.
- Esteller M, Garcia-Foncillas J, Andion E, et al. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *N Engl J Med*. 2000;343(19):1350–1354.
- Stupp R, Gander M, Leyvraz S, Newlands E. Current and future developments in the use of temozolomide for the treatment of brain tumours. *Lancet Oncol*. 2001;2(9):552–560.
- Hegi ME, Diserens AC, Gorlia T, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med*. 2005;352(10):997–1003.
- Yoshino A, Ogino A, Yachi K, et al. Gene expression profiling predicts response to temozolomide in malignant gliomas. *Int J Oncol*. 2010;36(6):1367–1377.
- Weller M, Tabatabai G, Kästner B, et al. MGMT promoter methylation is a strong prognostic biomarker for benefit from dose-intensified temozolomide rechallenge in progressive glioblastoma: the DIRECTOR trial. *Clin Cancer Res*. 2015;21(9):2057–2064.
- Srivenugopal KS, Yuan XH, Friedman HS, Ali-Osman F. Ubiquitination-dependent proteolysis of O6-methylguanine-DNA methyltransferase in human and murine tumor cells following inactivation with O6-benzylguanine or 1,3-bis(2-chloroethyl)-1-nitrosourea. *Biochemistry*. 1996;35(4):1328–1334.
- Esquela-Kerscher A, Slack FJ. Oncomirs: microRNAs with a role in cancer. *Nat Rev Cancer*. 2006;6(4):259–269.
- Akao Y, Nakagawa Y, Naoe T. MicroRNAs 143 and 145 are possible common onco-microRNAs in human cancers. *Oncol Rep*. 2006;16(4):845–850.
- Chan JA, Krichevsky AM, Kosik KS. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. *Cancer Res*. 2005;65(14):6029–6033.
- Cowland JB, Hother C, Grønbaek K. MicroRNAs and cancer. *APMIS*. 2007;115(10):1090–1106.
- Iorio MV, Ferracin M, Liu CG, et al. MicroRNA gene expression deregulation in human breast cancer. *Cancer Res*. 2005;65(16):7065–7070.
- Wu L, Cai C, Wang X, Liu M, Li X, Tang H. MicroRNA-142-3p, a new regulator of RAC1, suppresses the migration and invasion of hepatocellular carcinoma cells. *FEBS Lett*. 2011;585(9):1322–1330.
- Deng B, Zhang Y, Zhang S, Wen F, Miao Y, Guo K. MicroRNA-142-3p inhibits cell proliferation and invasion of cervical cancer cells by targeting FZD7. *Tumour Biol*. 2015;36(10):8065–8073.
- Lei Z, Xu G, Wang L, et al. MiR-142-3p represses TGF- β -induced growth inhibition through repression of TGF β R1 in non-small cell lung cancer. *FASEB J*. 2014;28(6):2696–2704.
- Chiou GY, Chien CS, Wang ML, et al. Epigenetic regulation of the miR-142-3p/interleukin-6 circuit in glioblastoma. *Mol Cell*. 2013;52(5):693–706.
- Ujifuku K, Mitsutake N, Takakura S, et al. miR-195, miR-455-3p and miR-10a* are implicated in acquired temozolomide resistance in glioblastoma multiforme cells. *Cancer Lett*. 2010;296(2):241–248.
- Palanichamy JK, Rao DS. miRNA dysregulation in cancer: towards a mechanistic understanding. *Front Genet*. 2014;5:54.
- Shrestha A, Mukhametshina RT, Taghizadeh S, et al. MicroRNA-142 is a multifaceted regulator in organogenesis, homeostasis, and disease. *Dev Dyn*. 2017;246(4):285–290.
- Xiao P, Liu WL. MiR-142-3p functions as a potential tumor suppressor directly targeting HMGB1 in non-small-cell lung carcinoma. *Int J Clin Exp Pathol*. 2015;8(9):10800–10807.
- Chen Y, Zhou X, Qiao J, Bao A. MiR-142-3p overexpression increases chemo-sensitivity of NSCLC by inhibiting HMGB1-mediated autophagy. *Cell Physiol Biochem*. 2017;41(4):1370–1382.
- Liu K, Huang J, Ni J, et al. MALAT1 promotes osteosarcoma development by regulation of HMGB1 via miR-142-3p and miR-129-5p. *Cell Cycle*. 2017;16(6):578–587.
- Xu G, Wang J, Jia Y, Shen F, Han W, Kang Y. MiR-142-3p functions as a potential tumor suppressor in human osteosarcoma by targeting HMGA1. *Cell Physiol Biochem*. 2014;33(5):1329–1339.
- Chen Y, Hu F, Zhou Y, Chen W, Shao H, Zhang Y. MGMT promoter methylation and glioblastoma prognosis: a systematic review and meta-analysis. *Arch Med Res*. 2013;44(4):281–290.
- Grimson A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol Cell*. 2007;27(1):91–105.
- Jackson AL, Burchard J, Schelter J, et al. Widespread siRNA “off-target” transcript silencing mediated by seed region sequence complementarity. *RNA*. 2006;12(7):1179–1187.
- Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*. 2005;120(1):15–20.
- Mathonnet G, Fabian MR, Svitkin YV, et al. MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F. *Science*. 2007;317(5845):1764–1767.

34. Chi SW, Zang JB, Mele A, Darnell RB. Argonaute HITS-CLIP decodes microRNA-mRNA interaction maps. *Nature*. 2009;460(7254):479–486.
35. Seok H, Ham J, Jang ES, Chi SW. MicroRNA target recognition: insights from transcriptome-wide non-canonical interactions. *Mol Cells*. 2016;39(5):375–381.
36. Flamand MN, Gan HH, Mayya VK, Gunsalus KC, Duchaine TF. A non-canonical site reveals the cooperative mechanisms of microRNA-mediated silencing. *Nucleic Acids Res*. 2017;45(12):7212–7225.
37. Khalil S, Fabbri E, Santangelo A, et al. miRNA array screening reveals cooperative MGMT-regulation between miR-181d-5p and miR-409-3p in glioblastoma. *Oncotarget*. 2016;7(19):28195–28206.
38. Kushwaha D, Ramakrishnan V, Ng K, et al. A genome-wide miRNA screen revealed miR-603 as a MGMT-regulating miRNA in glioblastomas. *Oncotarget*. 2014(12):4026–4039.
39. Anda T, Shabani HK, Tsunoda K, et al. Relationship between expression of O6-methylguanine-DNA methyltransferase, glutathione-S-transferase pi in glioblastoma and the survival of the patients treated with nimustine hydrochloride: an immunohistochemical analysis. *Neurol Res*. 2003;25(3):241–248.
40. Brell M, Tortosa A, Verger E, et al. Prognostic significance of O6-methylguanine-DNA methyltransferase determined by promoter hypermethylation and immunohistochemical expression in anaplastic gliomas. *Clin Cancer Res*. 2005;11(14):5167–5174.
41. Gusyatiner O, Hegi ME. Glioma epigenetics: from subclassification to novel treatment options. *Semin Cancer Biol*. Epub 2017 Nov 21.
42. Blanc JL, Wager M, Guilhot J, et al. Correlation of clinical features and methylation status of MGMT gene promoter in glioblastomas. *J Neurooncol*. 2004;68(3):275–283.
43. Sadones J, Michotte A, Veld P, et al. MGMT promoter hypermethylation correlates with a survival benefit from temozolomide in patients with recurrent anaplastic astrocytoma but not glioblastoma. *Eur J Cancer*. 2009;45(1):146–153.
44. Taylor JW, Schiff D. Treatment considerations for MGMT-unmethylated glioblastoma. *Curr Neurol Neurosci Rep*. 2015;15(1):507.
45. Low SY, Ho YK, Too HP, Yap CT, Ng WH. MicroRNA as potential modulators in chemoresistant high-grade gliomas. *J Clin Neurosci*. 2014;21(3):395–400.

Cancer Management and Research

Publish your work in this journal

Cancer Management and Research is an international, peer-reviewed open access journal focusing on cancer research and the optimal use of preventative and integrated treatment interventions to achieve improved outcomes, enhanced survival and quality of life for the cancer patient. The manuscript management system is completely online and includes

Submit your manuscript here: <https://www.dovepress.com/cancer-management-and-research-journal>

a very quick and fair peer-review system, which is all easy to use. Visit <http://www.dovepress.com/testimonials.php> to read real quotes from published authors.

Dovepress