Combination treatment with doxorubicin and microRNA-21 inhibitor synergistically augments anticancer activity through upregulation of tumor suppressing genes

SHANSHAN ZHANG[1], LEI HAN[2-4], JIANWEI WEI[2-4], ZHENDONG SHI[2-4], PEIYU PU[2-4], JIANNING ZHANG[2-4], XUBO YUAN[1] and CHUNSHENG KANG[2-4]

[1]Tianjin Key Laboratory of Composite and Functional Materials, School of Materials Science and Engineering, Tianjin University, Tianjin 300072; [2]Laboratory of Neuro-Oncology, Department of Neurosurgery, Tianjin Neurological Institute, Tianjin Medical University General Hospital; [3]Key Laboratory of Neurotrauma, Variation, and Regeneration, Ministry of Education and Tianjin Municipal Government, Tianjin 300052; [4]Chinese Glioma Cooperative Group (CGCG), P.R. China

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Abstract. Doxorubicin (DOX) is a key chemotherapeutic drug for cancer treatment. The antitumor mechanism of DOX is its action as a topoisomerase II poison by preventing DNA replication. Our study shows that DOX can be involved in epigenetic regulation of gene transcription through downregulation of DNA methyltransferase 1 (DNMT1) then reactivation of DNA methylation-silenced tumor suppressor genes in glioblastoma (GBM). Recent evidence demonstrated that microRNA (miR or miRNA) can mediate expression of genes through post-transcriptional regulation and modulate sensitivity to anticancer drugs. As one of the first miRNAs detected in the human genome, miR-21 has been validated to be overexpressed in GBM. Combination treatment of a chemotherapeutic and miRNA showed synergistically increased anticancer activities which has been proven to be an effective strategy for tumor therapy. In our study, co-treatment of DOX and miR-21 inhibitor (miR-21i) resulted in remarkably increased expression of tumor suppressor genes compared with DOX or the miR-21i treatment alone. Moreover, we demonstrate that combining DOX and miR-21i significantly reduced tumor cell proliferation, invasion and migration *in vitro*. Our study concludes that combining DOX and miR-21i is a new strategy for the therapy of GBM.

Correspondence to: Professor Xubo Yuan, School of Materials Science and Engineering, Tianjin University, Tianjin 300072, P.R. China
E-mail: xbyuan@tju.edu.cn

Professor Chunsheng Kang, Laboratory of Neuro-Oncology, Tianjin Neurological Institute, Tianjin Medical University General Hospital, Tianjin 300052, P.R. China
E-mail: kang97061@yahoo.com

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Introduction

Glioblastoma (GBM) is the most common and lethal primary malignancy of the central nervous system. Even with surgical resection and aggressive treatment with chemo- and radiotherapy, the prognosis remains very poor. A wide variety of novel therapeutic approaches have been developed and are currently under study as potential treatments for GBM (1).

The anticancer drug doxorubicin (DOX) primarily exhibits a wide spectrum of cytotoxic effects (2). Its planar three-ring structure stabilizes the topoisomerase II-DNA cleavable complex by DNA intercalation and enhances cleavage of DNA at both strands in a topoisomerase II-dependent manner. DOX also reacts with cellular formaldehyde to form DNA adducts (3). To effectively utilize the antitumor function of DOX, there have been few studies focusing on the other mechanisms of DOX (4).

Epigenetic lesions in DNA without mutations in the coding regions have been shown to be common phenomena in the pathogenesis of GBM, especially the methylation-mediated silencing of tumor suppressor genes such as VHL, p16, E-cadherin, PTEN, p21 and RECK, MGMT, RASSF1A (5-8). When DNA is methylated in the promoter region of genes where transcription is initiated, genes are inactivated and silenced (9). The cancer methylome is highly disrupted, making DNA methylation an excellent target for anticancer therapies. Several small synthetic and natural molecules are thus able to reverse the DNA hypermethylation through inhibition of DNA methyltransferase (DNMT). Over the last few decades, an increasing number of DNMT inhibitors targeting DNA methylation have been developed to increase efficacy with reduced toxicity (10). Tumor suppressor gene inactivation has previously been correlated with DNMT1 overexpression in various types of cancers (11). Knockdown of DNMT1 can repress tumor suppressor genes (12). Previous studies have

clarified that DOX is likely to affect DNA methylation by inhibiting catalytic activity of DNMT1 (13). We therefore hypothesized that DOX might indirectly alter epigenetic regulation of gene expression.

MicroRNAs (miRNAs) are small non-coding RNAs that act through post-transcriptional silencing in critical regulatory roles in multiple cellular functions (14). MicroRNAs represent an abundant class of endogenously 18-25 nucleotide non-coding RNA molecules which silence gene expression through a process of post-transcriptional modification. As one of the first miRNAs detected in the human genome, miR-21 has been validated to be involved in many different types of human cancers. Through targeting of PTEN, PDCD4, RECK and other signal transduction pathways, miR-21 regulates the proliferation, apoptosis, and invasion of hepatocellular cancer, colorectal cancer, breast cancer (15-19). Thus, targeting miR-21 and inhibiting its activity may be emerging as a promising therapeutic option and offer a potential new mode of cancer therapy.

Currently, therapies which simultaneously administer small molecular chemotherapeutic drug with gene medicine are common and effective ways to treat cancer (20). Cheng *et al* demonstrated that the folate-targeted co-delivery of Bcl-2 siRNA and DOX system caused not only an obvious reduced expression of anti-apoptotic Bcl-2 gene but also a remarkable elevated level of the pro-apoptotic Bax gene, resulting in the significantly apoptosis in tumor tissues (21). Based on that, we hypothesized DOX and miR-21 inhibitor (miR-21i) could regulate gene expression synergistically to inhibit tumor cells.

In the above study, we detected the expression of tumor suppressor genes E-cadherin, RECK, PTEN, p21, VHL and miR-200a/b/429 and miR-181d to discuss the effect of DOX and miR-21i on GBM suppression. In our present study, we discovered that DOX caused not only downregulation of DNMT1 but also obviously upregulation of PTEN and p21 genes as well as 4 non-coding miRNAs in the transcription stage. At the same time, miR-21i can regulate gene expression through post-transcriptional regulation. In addition, we have shown that combining DOX and miR-21i enhanced methylation associated tumor suppressor gene expression, this synergetic effect took place at the transcriptional level and post-transcriptional level. Furthermore, co-treatment with DOX and miR-21i strengthened antitumor effect, resulting in reduced tumor cell migration and cell invasion *in vitro*.

Materials and methods

Reagents, cell culture and transfection. The antisense oligonucleotide sequence of 2'-O-methyl (2'-O-Me) miR-21 inhibitor was: 5'-GTC CAC TCT TGT CCT CAA TG-3'. A scrambled inhibitor sequence (5'-AAG GCA AGC UGA CCC UGA AGU-3') was used as the negative control. They were chemically synthesized by Shanghai Gene Pharma (Shanghai, China) and dissolved in diethylpyrocarbonate (DEPC) water and frozen at -20˚C. DOX hydrochloride was purchased from Sigma-Aldrich. It dissolved in PBS and diluted in serum-free medium.

Human glioma cell lines U87 (PTEN del/EGFR wt), U87 EGFRvIII (PTEN del/EGFR mut), LN229 (PTEN wt/EGFR wt) were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). The human GBM cell lines U87, U87 EGFRvIII, and LN229 were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% FBS (heat-inactivated fetal bovine serum) (Hyclone) at 37˚C, 5% CO_2. The miR-21 inhibitor was transfected with Lipofectamine 3000 (Invitrogen, Grand Island, NY, USA). Transfections with hsa-miR-21 inhibitor and scrambled inhibitor were performed in serum-free medium 24 h after plating. Cell transfection used Lipofectamine 3000 according to the manufacturer's instructions. For each 6-well, miRNA in 125 μl of serum-free medium was mixed with 5 μl of Lipofectamine 3000 in 125 μl of the same medium and allowed to stand at room temperature for 5 min. The mixture was then added to cells and after 6 h the medium was changed to complete medium.

Evaluation of DOX cytotoxicity. The cytotoxicity of DOX was evaluated by the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Five thousand cells/well were seeded in 96-well plates at 37˚C for 24 h in 100 μl DMEM, which was supplemented with 10% FBS, 2 mM of glutamine (Sigma), 100 mg/ml of penicillin (Sigma) and 100 mg/ml of streptomycin (Sigma). Cancer cells were exposed to different concentrations of DOX (0, 0.5, 1, 1.5, 2, 4, 8 and 10 μM/l) for 48 h. To assess cell viability, 20 μl of MTT (5 mg/ml) was added to each well and the cells were incubated at 37˚C for another 4 h. The reaction was then stopped by dissolving the cells in 200 μl of dimethyl sulfoxide (DMSO) with shaking for 15 min to dissolve the formazan crystals formed by the living cells. Quantification measurements (optical density) were obtained at a wavelength of 490 nm using spectrophotometric analysis. Cells without treatment were used as control.

Flow cytometric analysis of cellular uptake of DOX. Cells ($2x10^5$) were cultured in a 6-well plate at 37˚C for 24 h in 2 ml DMEM. Cancer cells were then exposed to different concentrations of DOX (0, 0.5, 1.5 and 8 μM/l) for 6 h. At the end of the incubation period, cells were trypsinized and washed three times with PBS then fixed and resuspended in 75% ethyl alcohol of the corresponding temperature. Uptake rates were detected via flow cytometry (Becton-Dickinson, USA). Cells were passed through a 37-μm nylon filter to ensure a single-cell suspension. Laser excitation was at 488 nm and fluorescence was detected at 575 nm. Files were collected of 20,000 gated events and analyzed with the FACS software program.

Confocal fluorescence microscopy analysis of DOX intracellular uptake and distribution. A confocal fluorescent microscopy was used to compare the intracellular uptake of DOX (excitation/emission: 480/575 nm) and to investigate their cellular distribution. Cells ($2x10^5$) were grown on glass cover slips in a 6-well plate at 37˚C for 24 h in 2 ml DMEM. Cancer cells were then exposed to different concentrations of DOX (0, 0.5, 1.5, 8 μM/l) for 6 h. At the end of the incubation period, the medium was removed and the cells were washed three times with cold PBS and then fixed in 4% paraformaldehyde for 30 min. The fixed cells were washed three times with PBS at room temperature on a shaker. For nucleus labeling, cells were incubated with Clear-Mount (aqueous) containing DAPI (excitation/emission: 345/661 nm) for 10 min. The fluorescent

images of cells were analyzed using a laser scanning confocal microscope.

Protein extraction and western blot analysis. Glioma cells were treated with DOX/miR-21i alone or compound respectively. Cells were harvested 48 h after transfection. Each group of cells were then washed in cold PBS three times and then solubilized in 1% Nonidet P-40 lysis buffer. Homogenates were clarified by centrifugation at 12,000 rpm for 15 min at 4˚C, and protein concentrations were determined with Nanodrop spectrophotometer (Gene, USA). The protein contents of the lysates (50 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12, 10 and 8%, which were then transferred onto PVDF membranes (Millipore, Billerica, MA, USA). The membranes were probed overnight with primary antibodies against DNMT1 (1:1,000 dilution, Cell Signaling Technology, USA), E-cadherin (1:1,000 dilution, Abcam, UK), RECK (1:1,000 dilution, Santa Cruz, USA), PTEN (1:1,000 dilution, Santa Cruz), VHL (1:1,000 dilution, Abcam), p21 (1:1,000 dilution, Cell Signaling Technology) and GAPDH (1:1,000 dilution, Santa Cruz). The membranes were subsequently washed three times with PBS to remove excess primary antibodies, and incubated with appropriate HRP-conjugated secondary antibodies (1:1,000 dilution, Beijing Zhongshan Bio Corp., Beijing, China). GAPDH was selected as a housekeeping gene.

RNA extraction and real-time PCR. Total RNA was extracted from cultured cells with TRIzol reagent (Invitrogen) according to standard protocol. To detect the concentration of total mRNA, a nanodrop spectrophotometer (Gene) was utilized. Reverse transcription (RT) was conducted with the Go Scsipt™ Reverse Transcription System (Promega, USA). MJ-real time PCR (Bio-Rad, USA) was used to achieve the amplification reaction and the protocol was carried out for 40 cycles at 95˚C for 3 min, 95˚C for 15 sec and 60˚C for 30 sec. Both RT and PCR primers were purchased from Gene Pharma. The relative expression of miRNA/mRNA was evaluated via comparative CT (threshold cycle) and was normalized to the expression of U6/GAPDH RNA (Table I). All RT-PCR reactions were performed in triplicate.

Wound healing assay. Prior to wounding, cell culture and transfection conditions were optimized to ensure a homogeneous and viable cell monolayer. One day prior to transfection, equal quantities of GBM U87 and LN229 cells ($2x10^5$) were seeded in 6-well plates. The U87 and LN229 cells were treated with PBS, scrambled inhibitor, DOX, miR-21i or DOX/miR-21i, respectively. Cell transfection used Lipofectamine 3000 according to the manufacturer's instructions. When cell confluence reached ~90% at ~24 h post-transfection, an artificial homogenous wound was made to the monolayer using a sterile plastic 200 μl micropipette tip. Following wounding, debris was removed by washing cells three times with PBS. At different time-points, cells migrated into the wounded area or cells with extended protrusions from the wound border were photographed at x200 magnification under a light microscope.

Invasion assays. Invasive capacities of human GBM U87 and LN229 cells were tested via *in vitro* invasion assays (Becton-Dickinson Bio-Coat Matrigel Invasion Chamber). The top chamber of a transwell chamber was incubated with 60 μl Matrigel diluted with DMEM (1:2, Matrigel: DMEM) at 37˚C for 30 min. The Matrigel solidified and acted as an extracellular membrane (ECM) for tumor cell invasion analysis. The U87 and LN229 cells were treated with PBS, scrambled inhibitor, DOX, miR-21i or DOX/miR-21i, respectively. Cell transfection used Lipofectamine 3000 according to the manufacturer's instructions. After 24 h, each group of cells were adjusted to $5x10^5$/ml in DMEM and 100 μl of the resuspended cell solution was added to the top chamber over the Matrigel, with 100 μl of serum-free DMEM added up to 200 μl cell solution. The cells were induced to invade toward a chemoattractant filled with 500 μl of DMEM (with 10% FBS) which was placed into the lower chambers of the wells. The transwell plate was assembled and incubated at 37˚C, in a 5% CO_2 incubator. Following 24-h incubation, the non-invading cells were removed from the upper surfaces of the invasion membranes. Cells were stained by crystal violet for 3 min, and washed with PBS to remove excess stain. The chambers were gently scraped with a wet cotton swab. Images of each well were captured by microscopic analysis with an Olympus Vanox. All experiments were performed in triplicate.

Statistical analysis. All the experiments were carried out in triplicate and data were analyzed using Windows SPSS software. Quantitative values are expressed as means ± standard error, statistical analyses were performed using t-test. Differences were considered significant for $p \leq 0.05$. One-way ANOVA was used to test for differences among at least 3 groups, and the least significant difference post-hoc test was utilized to obtain individual p-values followed by ANOVA. The t-test was utilized to determine differences in each dual group comparison.

Table I. Representative gene primers utilized to perform quantitative PCR of mRNA.

Gene	Primer sequences (5'-3')
DNMT1	Forward: GGTGGAGAGTTATGACGAG
	Reverse: TAGAATGCCTGATGGTCTG
E-cadherin	Forward: TGATTCTCTGCTCGTGTT
	Reverse: CGTTCAAGTAGTCATAGTCC
RECK	Forward: GCTGTAGAAACCTTACTTACTG
	Reverse: GCTATTGCTTTCCACATCTC
PTEN	Forward: CTTCTACTGCCTCCAACAC
	Reverse: AGACGAATAATCCTCCGAAC
VHL	Forward: GTAGCGGTTGGTGACTTG
	Reverse: CCCTGGTTTGTTCCTCTG
p21	Forward: CCCTTGTCCTTTCCCTTC
	Reverse: GTGCCCTTCTTCTTGTGT
GAPDH	Forward: CCGGGAAACTGTGGCGTGATGG
	Reverse: AGGTGGAGGAGTGGGTGTCGCTGTT

Results

Modes of cell death induced by DOX. Human GBM cells were used to identify and characterize the various types of cell deaths induced by DOX. The cytotoxicity of the DOX was evaluated by MTT assay in human glioma cells. Human GBM cells LN229 (PTEN wt/EGFR wt), U87 (PTEN del/EGFR wt) and U87 EGFRvIII (PTEN del/EGFR mut) were first infected with different concentration gradient of DOX. We found that the three kinds of cell lines produced different reactions to the tested range of DOX concentrations (0.5-10 μM). As shown in Fig. 1A-a, LN229 cell line survival rates decreased gradually with the increase of drug concentrations. U87 and U87 EGFRvIII cell line survival rates, however, decreased radically in the low drug concentration (lower in 2 μM) but higher doses of DOX did not result in significant apoptosis, which appeared to be a relatively high platform on the survival curve under the conditions of relatively high drug concentrations. The survival rate was ~40%. The results reveal that PTEN or EGFR may affect the biological activity of DOX in tumor cells and lead to drug resistance. Several studies have shown that the signaling pathway activated by the lipid kinase phosphoinositide 3-kinase (PI3K) and the serine/threonine kinase, protein kinase B (PKB) or Akt, play a more important role in chemoresistance including DOX (22,23). Given that tumor suppressor gene PTEN is a negative regulator of the PI3K pathway, the most extensive evidence for the involvement of the PI3K pathway in human cancer stems from studies of the PTEN. Loss of PTEN can be sustained activation of this pathway. PI3K pathway activation contributes to the effects chemoresistance (24). On the other hand, the increasing of mutant EGFR receptor (EGFRvIII) expression lead to continuous activation of EGFR. EGFRvIII signaling also activates the PI3K pathway in glioblastoma cell lines (25,26). Thus, loss of PTEN or EGFR mutations may play an important role in glioblastoma cell chemoresistance to DOX. We believe this is worthy of further study.

miR-21i and DOX on proliferation of GBM cells. Previous studies have clarified that the expression of miR-21 was upregulated in human GBM. In order to examine whether miR-21 could modulate the chemosensitivity to DOX in GBM cells, we detected the expression of miRNAs in DOX-resistant cells. miR-21i was transfected after treatment of DOX in U87 and U87 EGFRvIII cell lines. Sensitivity to DOX was increased by the specific inhibition of miR-21 of which the maximal inhibition differed for the two GBM cell lines. The results showed that miR-21 downregulated Dox-resistant U87 cells when treated with DOX at concentrations ranging from 0.5 to 10 μM as measured by MTT assay (Fig. 1A-b), markedly enhanced cell death was observed. This increased sensitivity to DOX was not, however, observed in U87 EGFRvIII cells after downregulating miR-21 on high DOX concentration, but rather simply delayed the emergence of plateaus in high drug concentrations. These data suggest that the synergistic effect appeared at low concentrations, and inhibition of miR-21 could sensitize GBM cells to anticancer drug DOX.

Characterization of DOX uptake in tumor cells. We examined cellular uptake of DOX at different concentrations. Flow cytometry and confocal microscopy were performed to observe cellular uptake and distribution of DOX. We chose 0.5, 1.5 and 8 μM as the representative drug concentrations. Flow cytometry revealed that the U87 cellular uptake of DOX dramatically increased from 3.46 to 99.48%, as did the U87-EGFRvIII cell line, changing from 3.10 to 98.65% (Fig. 1B). The highest DOX concentration displayed the highest cellular uptake with 6-h incubation. These results were further confirmed by confocal microscopy. The majority of visible DOX fluorescence (red) was mainly in the nuclei. Significantly higher intracellular DOX fluorescence intensity was observed in the nucleus of the U87/U87-EGFRvIII cell lines with the increase of DOX concentration (Fig. 1C), indicating an increased uptake of DOX by these cells. Taken together, our data show that DOX is localized in the nucleus and the amount in the nucleus gradually increased with the increase of drug concentration. At the same time, it suggests that loss of PTEN or EGFR mutations may be a reason for GBM cell chemoresistance to DOX.

DOX may act as a demethylation drug reactivating DNA methylation-silenced tumor suppressor genes. Anticancer drug DOX exhibits a wide spectrum of cytotoxic effects primarily as a topoisomerase II α poison. However, recent studies indicated that topoisomerase II may not be the main target. Other cellular responses to doxorubicin have also emerged, including the inhibition of the DNMT1 (13). DNMT1 is the primary enzyme responsible for maintenance of DNA methylation on genomic DNA. To assess whether DOX could lead to demethylation and introduction of tumor suppressor genes through blocking DNMT1 expression, we first analyzed the expression of DNMT1. After treating three GBM cells with DOX for 48 h, western blot (Fig. 2A-a) and PCR (Fig. 2A-b) analysis indicated that the protein and mRNA expression levels of DNMT1 was clearly reduced. Reports have shown that epigenetic lesions in DNA without mutations in the coding regions are a common phenomenon in the tumorigenesis of GBM, especially the methylation-mediated silencing of tumor suppressor genes such as VHL, PTEN, E-cadherin, DAPK, MGMT, EMP3 and p21 (27). We then investigated whether DOX could affect tumor suppressor encoding genes associated with methylation such as E-cadherin, RECK, PTEN, VHL and p21. Western blot analysis indicated that certain protein expression was dramatically increased, including PTEN, and p21 (Fig. 2B). However, the expression levels of E-cadherin, RECK, and VHL were not obviously changed. In order to further confirm the role of tumor suppressor gene methylation influenced by DOX, we performed Real time-PCR analyses to measure the expression of mRNA. Similar to western blot analysis, the mRNA expression of PTEN, and p21 was dramatically increased (Fig. 2C). However, the expression levels of E-cadherin, RECK, and VHL were not markedly changed.

After it was ascertained that DOX can affect expression of some tumor suppressor genes, we investigated whether DOX could regulate tumor suppressor non-coding genes. The number of miRNAs with putative tumor suppressor functions undergoing promoter CpG island hypermethylation in human cancer is one of the most common causes of aberrant silencing. Recent reports have also indicated the presence of hypermeth-

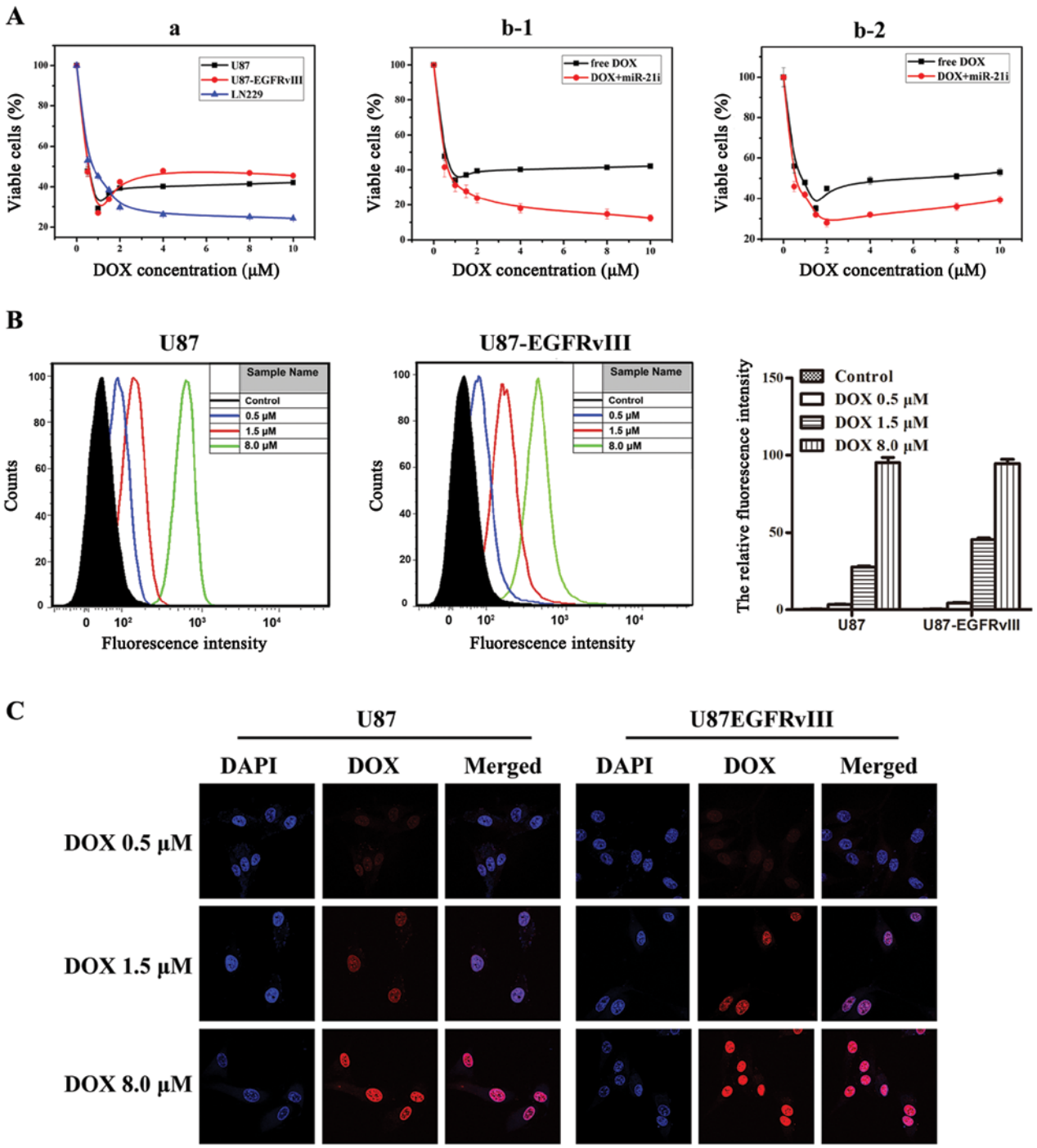

Figure 1. Effects of DOX/miR-21i on cell viability and characterization of DOX uptake by GBM cell lines. (A) Effect of DOX/miR-21i on cell viability: (a) U87, U87-EGFRvIII and LN229 GBM cell lines were treated with various concentrations of DOX (0, 0.5, 1, 1.5, 2, 4, 8 or 10 μM) and after 48-h incubation an MTT assay was performed. The viability of the untreated cells was regarded as 100%. Combination of DOX and miR-21i in the U87 (b-1) and U87-EGFRvIII (b-2) cell lines. (B) Cellular uptake of DOX detected by flow cytometry. (C) Cellular distribution of DOX detected by confocal microscopy. The two cell lines U87 and U87-EGFRvIII were treated with DOX (0, 0.5, 1.5 and 8.0 μM) in a 6-h incubation at 37˚C. Cells were counter-stained with DAPI (for nuclei), DOX (red fluorescence). Error bars represent the mean ± SD obtained from 3 independent experiments.

ylation-associated silencing of some miR-200 family members in cancer cells, and most importantly, the DNA methylation associated silencing of the miR-200 family determines the evolving epithelial-mesenchymal transition phenotypes (28). miR-181d was downregulated in human GBM, and acts as a tumor suppressor in GBM by targeting K-ras and Bcl-2 (29). Furthermore, our recent study demonstrated that regulation of Wnt/β-catenin signaling pathway affected GBM proliferation, and migration (30). We chose miRNAs which associated with tumor invasion: miR-200a/b/429, and miR-181d. Forty-eight hours later, the expression levels of miR-200a/b/429 and miR-181d were increased in varying degrees (Fig. 2D). This indicated that DOX can promote expression of tumor suppressor miRNAs.

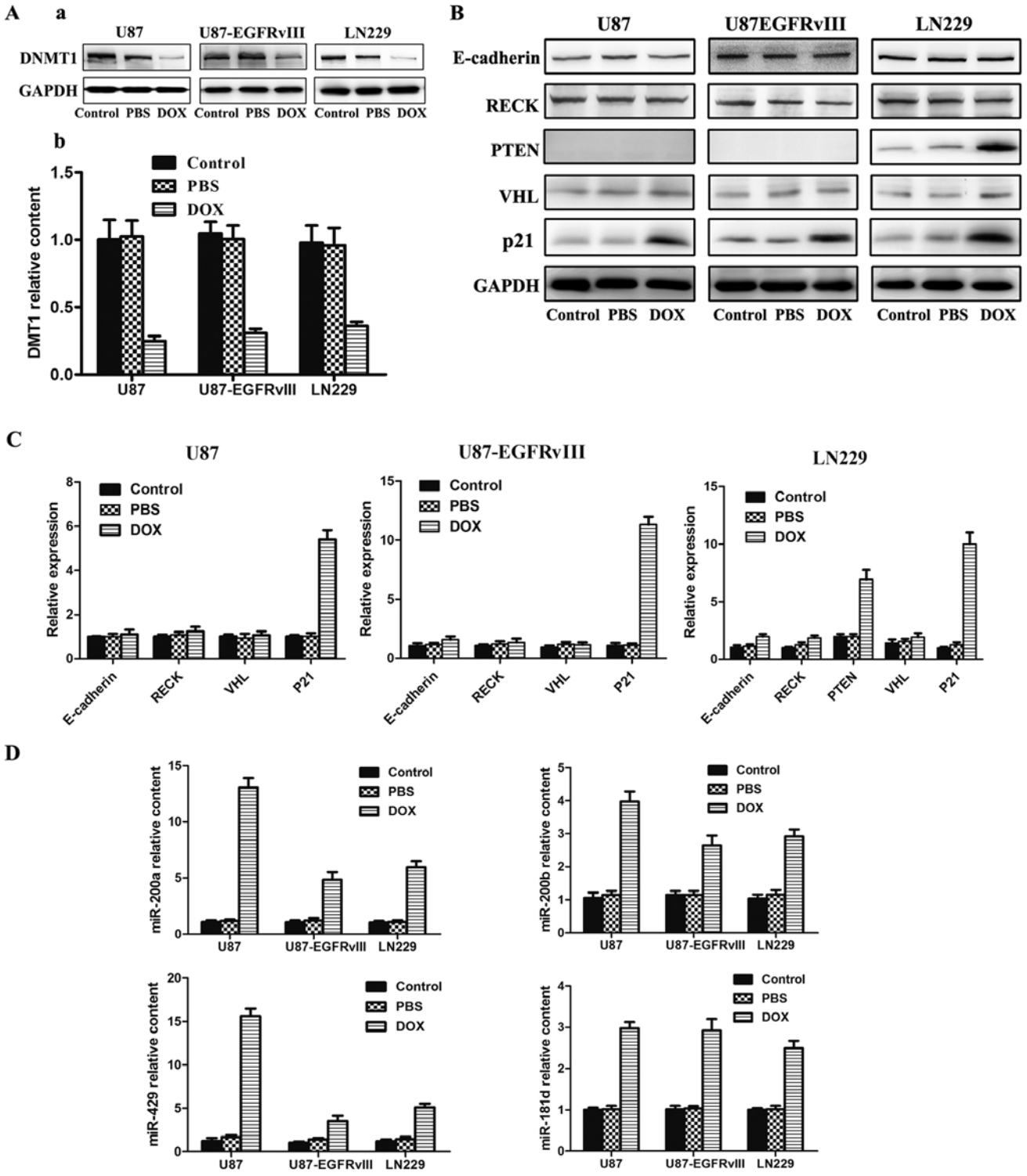

Figure 2. Effects of DOX on the functions of U87, U87-EGFRvIII and LN229 cell lines. (A) DNMT1 relative expression assayed using western blotting and RT-PCR. (B) Western blot detection of E-cadherin, RECK, PTEN, VHL and p21 expression. (C) RT-PCR analysis of E-cadherin, RECK, PTEN, VHL and p21 mRNA expression. (D) RT-PCR analysis of 4 miRNAs expression in the indicated cells. The cell lines were treated with DOX for 48 h at the concentration of 0.4, 0.4 and 0.5 μM respectively. Western blotting, GAPDH was used as the loading control. PCR, error bars represent the mean ± SD obtained from 3 independent experiments.

Global changes in gene expression induced by miR-21i in GBM cells. MiRNAs are small endogenous non-coding RNAs which downregulate gene expression primarily by binding to the 3'UTR of the target gene region (31). MicroRNA-21 negatively regulates several targets, and thus impacts tumorigenesis. Several targets of miR-21 have been experimentally validated, including PTEN, VHL and RECK. Ectopic expression of these targets may exert differing functional effects on tumorigenesis. All three cell lines were infected with miR-21i for 48 h. We then similarly investigated tumor suppressor encoding genes

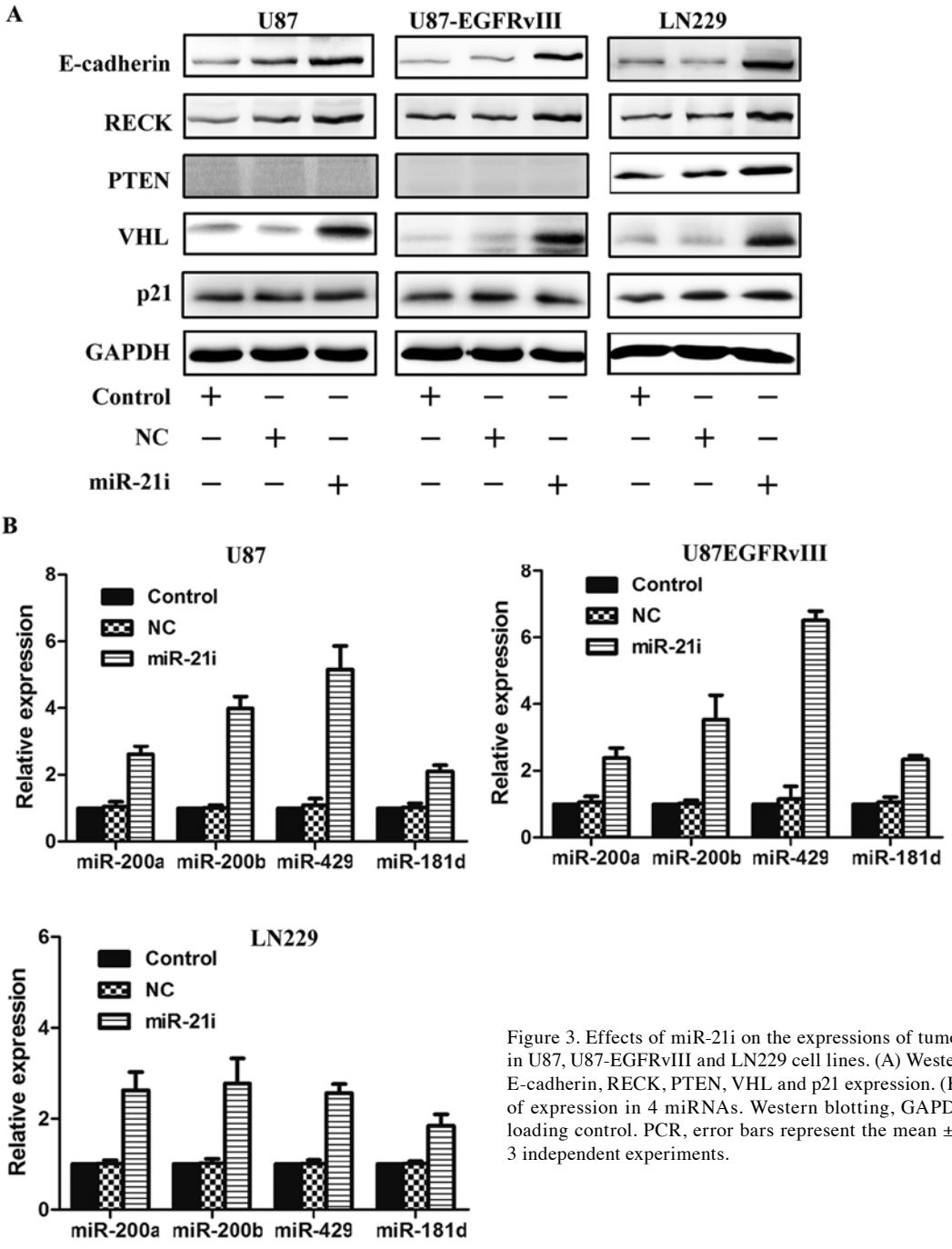

Figure 3. Effects of miR-21i on the expressions of tumor suppressor genes in U87, U87-EGFRvIII and LN229 cell lines. (A) Western blot detection of E-cadherin, RECK, PTEN, VHL and p21 expression. (B) RT-PCR analysis of expression in 4 miRNAs. Western blotting, GAPDH was used as the loading control. PCR, error bars represent the mean ± SD obtained from 3 independent experiments.

E-cadherin, RECK, PTEN, VHL, p21 and tumor suppressor non-coding genes miR-200a/b/429, miR-181d. Western blotting of the infected cells showed that the protein levels were increased for E-cadherin, RECK, PTEN, VHL, but p21 was virtually unchanged (Fig. 3A). Furthermore, PCR results revealed that expression levels of tumor suppressor miR-200a/b/429 and miR-181d were increased (Fig. 3B). These results provided evidence that downregulation of miR-21 could play the role of inhibiting GBM through upregulation tumor suppressor genes.

Co-treatment of DOX and miR-21 inhibitor enhances tumor suppressor genes expression. Although effective, DOX can promote expression of tumor suppressor genes and miRNA can regulate expression of tumor suppressor genes, single transfection DOX or miR-21 inhibitor is not very efficient in enhancing tumor-suppressor gene expression. We suspect that the combination DOX and miR-21 inhibitor can enhance the expression of tumor suppressor genes. To further investigate the expression level of coding genes and non-coding genes, three GBM cell types were simultaneously co-transfected with DOX and miR-21i. Consistent with our hypothesized results, western blotting revealed that protein levels of coding genes E-cadherin, RECK, PTEN, VHL and p21 were markedly increased in the co-treatment group as compared with the DOX or the miR-21i group alone (Fig. 4A). Furthermore, in the combined treatment, a significant inducement of the miRNAs (miR-200a/b/429, miR-181d) was observed with a greater level

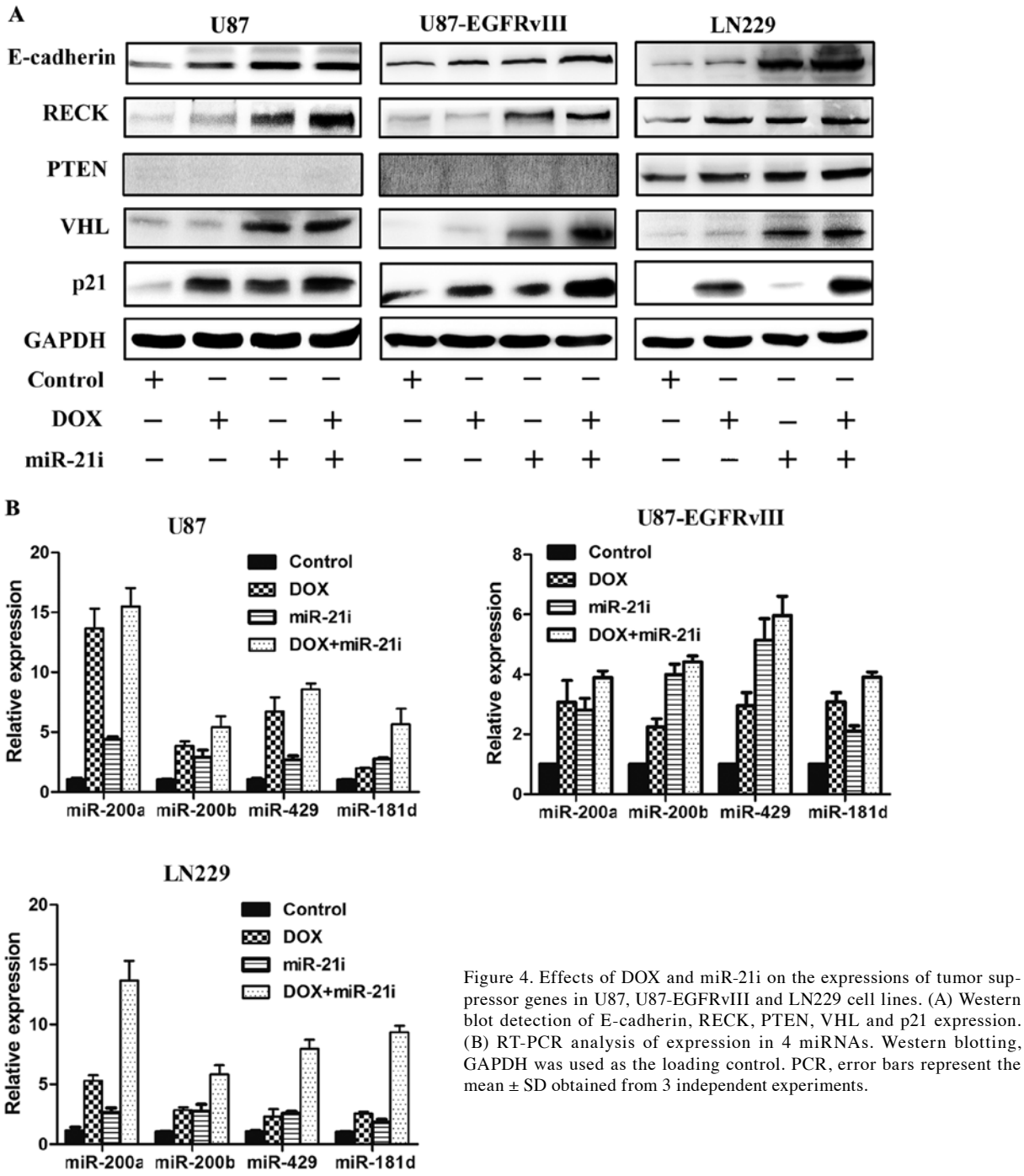

Figure 4. Effects of DOX and miR-21i on the expressions of tumor suppressor genes in U87, U87-EGFRvIII and LN229 cell lines. (A) Western blot detection of E-cadherin, RECK, PTEN, VHL and p21 expression. (B) RT-PCR analysis of expression in 4 miRNAs. Western blotting, GAPDH was used as the loading control. PCR, error bars represent the mean ± SD obtained from 3 independent experiments.

than that of DOX or miR-21i treatment alone, as shown in Fig. 4B. In addition, the treatment with DOX in combination with miR-21i induces a highly synergistic effect that upregulates tumor suppressor genes in GBM cells.

Regulation of tumor cell activity by DOX and miR-21i in vitro. Due to the aggressive growth characteristics of GBM, we investigated the regulation of tumor cell activity by DOX and miR-21i. Our previous study showed that elevated miR-200a inhibited cell growth and invasion through the Wnt/β-catenin pathway *in vitro* and *in vivo* (32,33). We likewise know about miR-181d mediated suppression of β-catenin/Wnt signaling (30). The expression level of miR-200 family/miR-181d clearly influenced the biological activity of GBM cells. We therefore investigated two major biological activities of tumor cells migration, and invasion potential. Migration and invasion potential are important biological characteristics of malignant tumor cells. To examine the cell migration *in vitro*, the scratch assay was employed, and more decreased mobility was observed in the DOX and miR-21i group (Fig. 5A). This result indicated an inhibitory effect of DOX and miR-21i on the migration ability. The number of U87 and LN229 cells invading through the Matrigel in the DOX and miR-21i group was significantly decreased compared to single DOX, miR-21i, control, PBS and scrambled inhibitor group (Fig. 5B). In summary, our results demonstrated that combination treat-

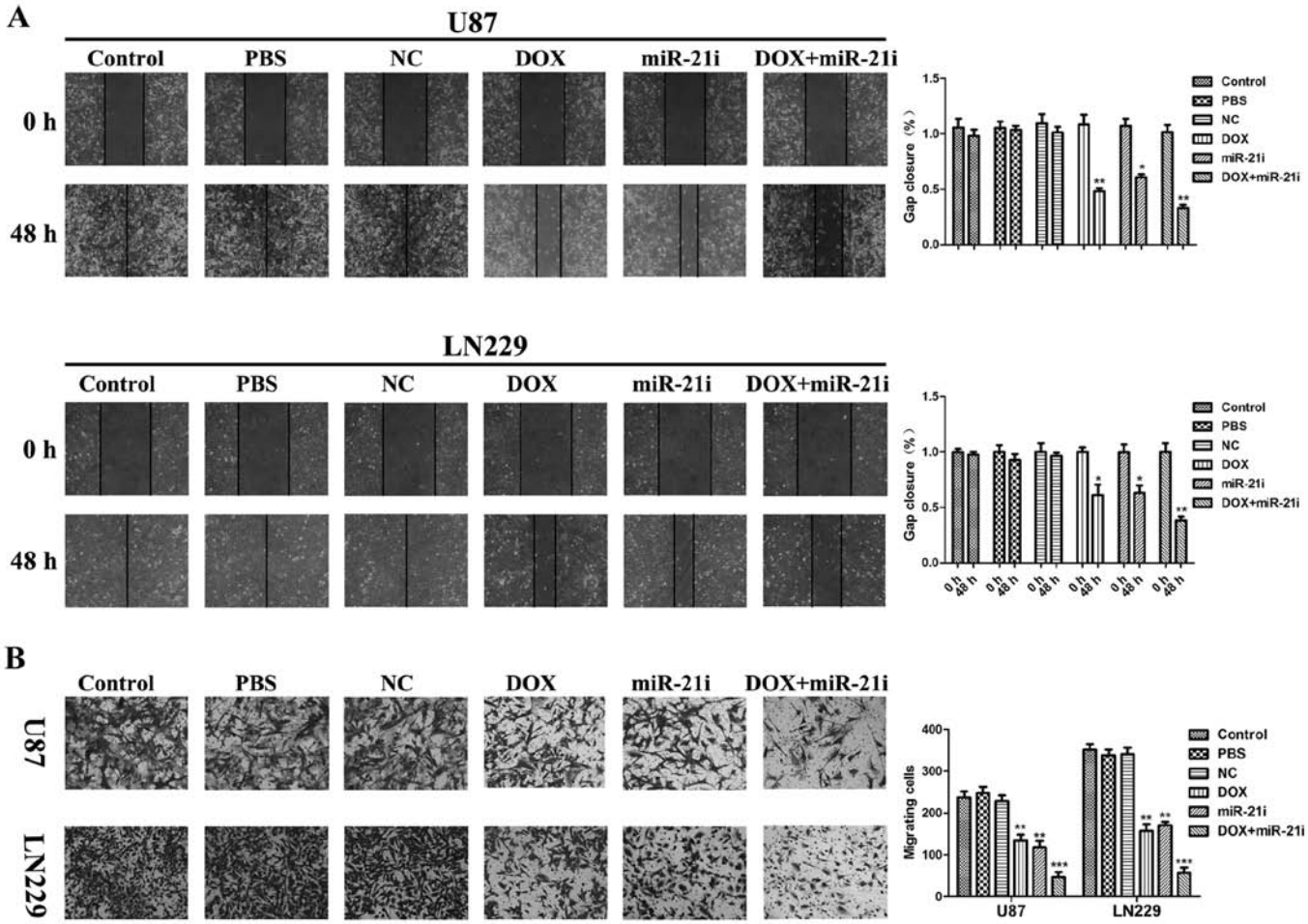

Figure 5. DOX and miR-21i affect migration and invasion of U87 and LN229 cell lines. Measurement of cell migration and invasion by 'wound-healing' assay and Transwell assay. All cell lines (U87 and LN229) were treated with PBS, scrambled inhibitor, DOX, miR-21i, DOX and miR-21i, respectively. (A) The cell migratory capabilities were assessed by wound-healing assay. The images were acquired immediately after scratching and 48 h later. (B) The Transwell assay indicated the decreased invasive capability of the combination treatment as compared to the DOX or miR-21i alone. Data shown are the mean and standard deviation from analyzing three fields per sample in triplicate ($^{*}p<0.05$, $^{**}p<0.01$).

ment with DOX and miR-21i significantly reduced tumor cell migration and cell invasion compared with DOX or the miR-21i treatment alone.

Discussion

The occurrence of GBM is associated not only with genetic changes but also with epigenetic alterations such as aberrations in DNA methylation patterns (27). Almost half of tumor-suppressor genes have been shown to be transcriptionally silenced (34). Silencing by DNA hypermethylation in GBM affects genes involved in key cellular functions such as the cell cycle (p16 and p21), tumor suppression (VHL and PTEN), DNA repair, and genome integrity (MGMT and MLH1) as well as tumor invasion and apoptosis (CDH1 and RECK) (7,27,35,36). For non-coding genes, miRNAs induce heritable changes in gene expression without altering DNA sequences and thus contribute to the epigenetic landscape. Therefore, CpG island promoter hypermethylation is one of the causes of the silencing of tumor suppressor miRNAs with a similar chromatin context to coding genes (37). Because cytosine methylation within the promoter regions of genes can cause transcriptional silencing, demethylation may activate the expression of genes that activate tumor suppressors in turn. Evidence has proved that anticancer drugs with DOX can act as DNA hypomethylating agents. According to Hanafy *et al* (4), total methylation percentage was markedly reduced from 62.2 (control) to 36.7% by the action of DOX. SP1049C, a Pluronic-based micellar formulation of DOX, significantly increased gene promoter demethylation compared to saline control P388 cancer stem cells *in vivo* (38). These results suggest that anticancer drug DOX may act as DNA hypomethylating agent.

DNMT1 is the best studied methyltransferase responsible for maintaining DNA methylation patterns in genomic DNA during DNA replication. High levels of DNMT1 expression have been reported to transcriptionally silence tumor suppressor genes (39,40). Since DNMT1 promotes methylation of DNA and is a key factor in maintaining DNA methylation, some recent research was committed to detect or downregulate DNMT1 (41,42). Further studies revealed DOX can interact with DNA including the formation of doxorubicin-DNA adducts, occur primarily at CpG sequences, then inhibit the DNA methyltransferase DNMT1 (13). Inactivation of glutamic

acid decarboxylase 67 (GAD67) and reelin might be due to the aberrant methylation of promoter-associated CpG islands. The doxorubicin decreased levels of DNMT1 were previously reported exploited to actively repress the GAD67 and reelin promoter eventually significantly increasing expression of reelin and GAD67 (43). Furthermore, knockdown of DNMT1 expression caused an increase in chemosensitivity toward cisplatin (39). Based on the ability of DOX to intercalate DNA and DNMT1 it can be recruited to DNA damage sites (44). We therefore hypothesized that DOX might act as a potential demethylating agent like 5-Aza-2'-deoxycytidine. In this study, E-cadherin, RECK, PTEN, VHL, p21 coding genes were demonstrated to be silenced and associated with the tumor invasion miRNAs (miR-200a/b/429, miR-181d non-coding genes). In the present study, we showed for the first time that the protein and mRNA expression of DNMT1 were reduced and the expression of PTEN and p21 was increased after DOX exposure in GBM cells. Rajendran *et al* (45) found that PTEN and p21 gene promoters displayed hypermethylation in the glioma cell lines. Downregulation of DNMT1 with DNMT inhibitor 5-azacytidine consequentially increase the expression of PTEN and p21, as shown through MS-PCR study. Furthermore, 4 miRNAs were similarly induced after DOX treatment. Lujambio *et al* (46) showed that some miRNAs were upregulated in a DNMT1 and DNMT3B double knockout in colon cancer cell line model. Davalos *et al* (28) found that treatment with the DNMT agent 5'-aza-2'-deoxycytidine in the miR-200 family hypermethylated cancer cell lines with the DNA-demethylating agent 5'-aza-2'-deoxycytidine increase the expression of the miRNAs. Although it remains to be confirmed in future experiments, DOX could detect changes in promoter methylation of tumor suppressor genes. These data indicated that DOX could block expression of DNMT1 resulting in re-expression of certain silencing genes and recovering the function of some tumor suppressor genes. Although the precise mechanism for this remains unclear, here we propose a potential mechanism of DOX in methylation. However, the expression of E-cadherin, RECK and VHL gene mRNA were very weak. Single DOX cannot effectively influence tumors. Neoplasms of high malignancy and metastasis are still considered incurable. Therefore, there is an urgent need to develop potential schemes for cancer treatment.

Currently, miRNA-based gene therapy offers the theoretical appeal of targeting multiple gene networks and has garnered increasing attention (47). Previous studies have clarified that the expression of miR-21 was upregulated in human GBM tissues as well as in other cancers (48,49). Previous studies have indicated that miR-21 was shown to regulate the proliferation, apoptosis, and invasion of glioma by directly targeting E-cadherin, PTEN, VHL and RECK (50-52). In our study, we demonstrated that downregulation of miR-21 could upregulate the expression of E-cadherin RECK, PTEN, VHL and a set of miRNAs including miR-200a/b/429 and miR-181d in U87, U87EGFRvIII, LN229 cells.

Regardless of whether DOX or miR-21i alone can upregulate the tumor suppressor genes, DOX plays a role of methylation in the stage of transcription regulation of gene expression. Furthermore, miRNAs act as post-transcriptional gene regulators. We therefore postulate a combination of drug and gene through regulation of the transcription and post-transcription to simultaneously regulate the expression of tumor suppressor genes. Our previous studies showed that miR-21i enhanced GBM cells sensitivity to Taxol, 5-FU and TMZ demonstrating miR-21 plays a critical role in drug chemosensitivity (53-55). In-depth studies still need to be performed to confirm the effects of the combined miR-21 inhibitor and DOX. The results of western blotting and PCR corresponded well with the fact that DOX and miR-21 can enhance expression of tumor suppressor genes.

It is well known that the process of epithelial-to-mesenchymal transition (EMT), characterized by loss of intercellular adhesion and polarity, cytoskeletal reorganization that enhances cell motility, and degradation of the basement membrane has been associated with tumor progression and metastasis (56). miR-200 family has been shown to regulate EMT by downregulating the expression of the transcriptional ZEB factors. Previous studies demonstrated that miR-200a regulates EMT through direct targets of β-catenin and ZEB in glioblastoma and gastric adenocarcinoma (32,33) and miR-181d was believed to be a tumor suppressor in GBM directly targeting Wnt/β-catenin signaling promoting tumor proliferation, migration and invasion (30). miR-21 can induce EMT during TGF-β in many tumor cell lines (57). In addition, our previous studies have verified that PTEN inhibits β-catenin activation via downregulation of pAKT (58), so the expression levels of PTEN may impact the EMT process via the regulation of β-catenin. We further validated the upregulation of RECK had a significant impact on tumor growth and invasion *in vitro* and *in vivo* (51). In our study, we demonstrated that co-treatment DOX and miR-21i can upregulate the expression of PTEN, RECK, miR-200a/b/429 and miR-181d to suppress tumor migration and invasion activity. The combination DOX and miR-21i may facilitate the inhibition of EMT in GBM.

In the present study, we provide evidence that DOX may be involved in epigenetic regulation of transcriptional activity of tumor suppressor genes in GBM. Whether the analogue can lead to hypomethylation of gene promoters wait further testing. Here we hypothesized changes in DNMT1 protein and mRNA levels in conjunction with DOX acting as a hypomethylation agent. We have demonstrated that the combination of DOX and miR-21i can enhance the expression of tumor suppressor genes compared to treatment alone. Additionally, we have shown combination treatment improved the cytotoxicity of DOX and decreased the migration, invasion abilities of the tumor cells, and demonstrated synergistic effect of anti-glioma *in vitro*.

In conclusion, DOX may promote expression of tumor suppressors through demethylation to inhibit development of tumor cells and miR-21 as microRNA in oncogenesis enhancing tumor cell sensitivity to DOX so as to improve anticancer effect, which may come into play at the post-transcriptional stage. Our study unravels a new important element of DOX anticancer activity and demonstrates its potential applications in demethylation. This study provides novel insights into the mechanisms of chemotherapeutic drug DOX with miRNA in regulation of tumor genes. This will offer a strong rationale for therapeutic applications involving cancer in the future. Further studies are under way to investigate new delivery to co-deliver DOX and miRNA to overcome blood-brain barrier (BBB) and enhance chemosensitivity.

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