

## miR-374a Inhibitor Enhances Etoposide-Induced Cytotoxicity Against Glioma Cells Through Upregulation of FOXO1

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Glioma is a commonly diagnosed brain tumor that shows high mortality rate. Despite the great advancement of cancer therapy in recent years, chemotherapy is still an important approach for treatment of glioma. However, long-term chemotherapy usually causes serious side effects or complications. It is desirable to take strategies to enhance the efficacy of current chemotherapy. In the present study, we observed obvious upregulation of miR-374a in glioma cells. More importantly, we found that knockdown of miR-374a was able to enhance the etoposide-induced cytotoxicity against glioma cells. Mechanically, we demonstrated that FOXO1 was the target of miR-374a in glioma. Treatment with miR-374a inhibitor induced overexpression of FOXO1, and thus promoted the expression of Bim and Noxa. Since Bim and Noxa act as key proapoptotic proteins in mitochondrial apoptosis, miR-374a inhibitor was able to enhance the etoposide-induced apoptosis pathway in glioma.

**Key words:** Etoposide; miR-374a inhibitor; Glioma; FOXO1

### INTRODUCTION

Glioma is a commonly diagnosed malignant cancer that shows poor prognosis and low survival rate. Nowadays, surgery resection and chemotherapy are still the dominant strategy for treatment of glioma<sup>1-3</sup>. However, long-term chemotherapy usually causes serious side effects or complications. It is urgent to inhibit the chemoresistance of glioma cells against chemotherapeutic drugs to obtain an efficient chemotherapy.

Etoposide (VP-16) is one kind of DNA topoisomerase II inhibitor that induces DNA damage and apoptosis. As a broad-spectrum antitumor chemotherapeutic drug, VP-16 is used for treatment of various cancers including glioma<sup>4,5</sup>. However, under the stress of continuous exposure to VP-16, glioma cells usually develop some mechanisms to survive under glioma treatment<sup>6,7</sup>. Some novel adjunctive therapies are required to reduce the chemoresistance to VP-16 and enhance the efficacy of current chemotherapy.

Recently, studies demonstrated that expression profile of microRNAs (miRNAs) is associated with chemosensitivity in cancers<sup>8,9</sup>. miRNAs are a class of endogenously expressed and noncoding small RNAs that suppress gene expression by binding to the 3'-untranslated region

(3'-UTR) of targeted mRNAs. Therefore, miRNAs participate in various biological processes such as cell proliferation, differentiation, metabolism, and apoptosis in cancer cells<sup>10-12</sup>. Moreover, it has been reported that dysregulation of miRNAs in cancer cells may induce serious chemoresistance. Correction of miRNA disorder can be used as a powerful approach to improve the efficiency of chemotherapy<sup>13-15</sup>. In the present study, we investigated the expression profile of miR-374a in glioma. We then studied the potential role of miR-374a in the VP-16 treatment for glioma.

### MATERIALS AND METHODS

#### Cell Lines and Patients' Specimens

Human glioma cell lines A172, U118-MG, and LN-18 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured at 37°C in Dulbecco's modified Eagle's Medium (DMEM; Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) in a humidified 5% CO<sub>2</sub> incubator. To investigate the role of miR-374a in the required resistance of VP-16 in glioma cells, we pretreated the A172 cells with 0.1 μM VP-16 (Sigma-Aldrich, Darmstadt, Germany) for 2 weeks, 4 weeks, or 6 weeks before

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detection of cell viability. These VP-16 pretreated A172 cells were named as A172-2W, A172-4W, and A172-6W, respectively. Human glioma tissues and their corresponding paracancerous tissues were obtained from 25 primary glioma patients (age range from 41–81 years, average age is 62 years) and collected at The Third Affiliated Hospital of Kunming Medical University between March 2016 and October 2017. We obtained informed consent from all patients. The experimental protocols were approved by the ethics committee of The Third Affiliated Hospital of Kunming Medical University.

#### *Quantitative Reverse Transcriptase Real-Time PCR (qRT-PCR)*

Total RNAs were extracted from patients' tissues and human glioma cell lines A172, U118-MG, and LN-18 using TRIzol® reagent (Invitrogen, Waltham, MA, USA). For analysis of miR-374a and forkhead box O 1 (FOXO1) expression, total RNAs were reverse transcribed using stem-loop RT primers and the PrimeScript RT reagent kit according to the manufacturer's instruction (Takara Bio, Inc., Otsu, Japan). The stem-loop RT primer of miR-374a is as follows: 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCACTTAGC-3'. SYBR® Premix Ex Taq II reagent (Takara Bio, Inc.) was used for PCR amplification of miR-374a and FOXO1 on an Applied Biosystems 7900HT thermocycler (Thermo Fisher Scientific, Waltham, MA, USA). Expression of miR-374a and FOXO1 was normalized to U6 snRNA and GAPDH, respectively. The relative expression was determined according to the  $2^{-Ct}$  analysis<sup>16</sup>.

#### *Transfection*

For knockdown of FOXO1, FOXO1 siRNA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For enforced expression of FOXO1, the recombinant pcDNA3.1 plasmid (Invitrogen) containing FOXO1 open reading frame was conducted. For transfection, 2 µg/ml of FOXO1 plasmid, 50 pmol/ml of FOXO1 siRNA, 50 pmol/ml of miR-374a mimic (5'-AUAUAAUACAA CCUGCUAAGUG-3'; GenePharma Co., Ltd., Shanghai, P.R. China), 50 pmol/ml of miR-374a antisense oligonucleotide (miR-374a inhibitor; 5'-CACUUAGCAGG UUGUAUUAUUAU-3'; GenePharma Co., Ltd.), and 50 pmol/ml of negative control oligonucleotide (NCO; 5'-UAAGUACAAUUAUGCGCCAAU-3'; GenePharma Co., Ltd.) were transfected into the glioma cell lines using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instruction.

#### *Cell Viability Assay*

Transfected A172 glioma cells were seeded in 96-well plates at a density of  $5 \times 10^3$  per well overnight at 37°C. Subsequently, the cells were treated with

different concentrations of VP-16 for 48 h. Cell viability was evaluated using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) assay as previously described<sup>17</sup>. The absorbance was read at 570 nm using a microplate reader. The half-maximal inhibitory concentration (IC<sub>50</sub>) of VP-16 was calculated according to the cell viability curve of A172 cells.

#### *Luciferase Reporter Assay*

FOXO1 3'-UTR containing putative miR-374a binding site was cloned into the downstream of firefly luciferase gene in the pMIR-REPORT™ miRNA Expression Reporter Vector (Thermo Fisher Scientific, Inc) and named as pMIR-WT FOXO1. The mutant FOXO1 reporter was created by mutating the seed region of the miR-374a binding site of pMIR-WT FOXO1 using the site-directed mutagenesis kit (Takara Bio, Inc.) and named as pMIR-MT FOXO1. To perform the luciferase reporter assay, A172 cells were incubated in 48-well plates overnight at 37°C. Subsequently, the cells were cotransfected with luciferase pMIR-REPORT, *Renilla* luciferase pRL-TK vectors (Promega Corporation, Madison, WI, USA), and miR-374a mimic/inhibitor by using Lipofectamine 2000. After 48 h of transfection, the luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega Corporation) according to the manufacturer's instruction.

#### *Immunoprecipitation*

Cells were collected and lysed with cold RIPA lysis buffer (Cell Signaling Technologies, Danvers, MA, USA) for 15 min at 4°C. Supernatant was then collected after centrifugation at 12,000 × g for 15 min. Next, primary antibodies were added into the protein supernatant and slowly shaken on a rotating shaker at 4°C overnight. Subsequently, protein A/G plus-Agarose beads (Santa Cruz Biotechnology) were added into the protein lysates for 1 h at 4°C. Immunoprecipitated pellets were then mixed with the SDS loading buffer and boiled for use in Western blot analysis.

#### *Western Blot Analysis*

Cells were lysed in the RIPA lysis buffer. Subsequently, 50 µg of total proteins were separated by 12.5% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). The membranes were then incubated with the primary antibodies [anti-FOXO1, GAPDH, Bim, Noxa, MCL-1, Bax, cytochrome c (cyto c), apoptosis-inducing factor (AIF), Cox IV, caspase 9, and caspase 3; Cell Signaling Technology] overnight at 4°C. Next, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h followed by detection with an enhanced chemiluminescence detection kit (Pierce, Rockford, IL, USA). In

addition, to evaluate the release of cyto c and AIF from the mitochondria into the cytoplasm, cellular mitochondria were separated using Mitochondria/Cytosol Fraction Kit (BioVision, Milpitas, CA, USA) according to the manufacturer's instruction. The remaining cell lysate was used as the cytoplasm fraction.

#### Flow Cytometry

For detection of mitochondrial membrane potential (MMP), A172 cells were collected and stained with JC-1 (Molecular Probes, Waltham, MA, USA) as an indicator<sup>18</sup> before analysis of MMP on flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA) according to the manufacturer's instruction. For measurement of apoptotic rate, A172 cells were collected and stained with Annexin-V/propidium iodide (PI; Sigma-Aldrich). Subsequently, cell apoptosis was analyzed by flow cytometry, and the annexin V<sup>+</sup> cells were considered as the apoptotic cells.

#### Statistical Analysis

Data are represented as mean  $\pm$  standard deviation (SD) and obtained from three independent experiments. For comparison analysis, two-tailed Student's *t*-tests were used to estimate the statistical differences between two groups. In addition, one-way ANOVA and Bonferroni post hoc test were used to determine the differences. Statistical analysis was performed using SPSS 14.0 software. A value of  $p < 0.05$  was considered to indicate a statistically significant difference.

## RESULTS

### miR-374a Is Overexpressed in Glioma Cells

To investigate the potential role of miR-374a in glioma, 25 primary glioma patients' tumor tissues and their paracancerous tissues were collected and analyzed. We found

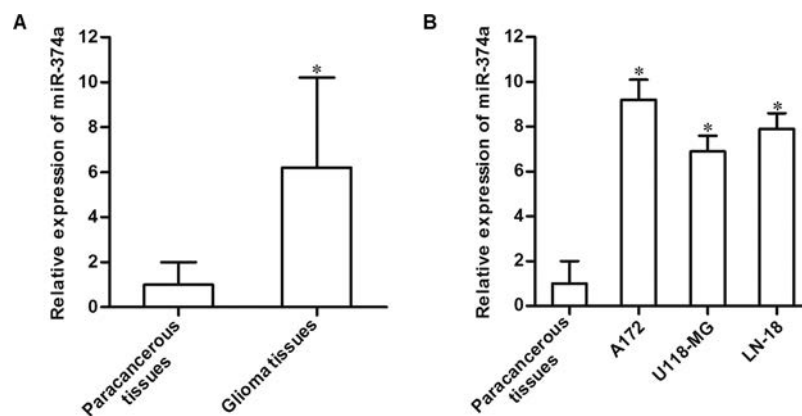
that expression level of miR-374a in glioma tissues was significantly higher than that in paracancerous tissues (Fig. 1A). We then analyzed the miR-374a expression in glioma cell lines. Our results showed obvious upregulation of miR-374a in A172, U118-MG, and LN-18 glioma cell lines in vitro (Fig. 1B). Taken together, we demonstrated that miR-374a was overexpressed in glioma cells.

### Knockdown of miR-374a Increases the Sensitivity of Glioma Cells to VP-16 Treatment

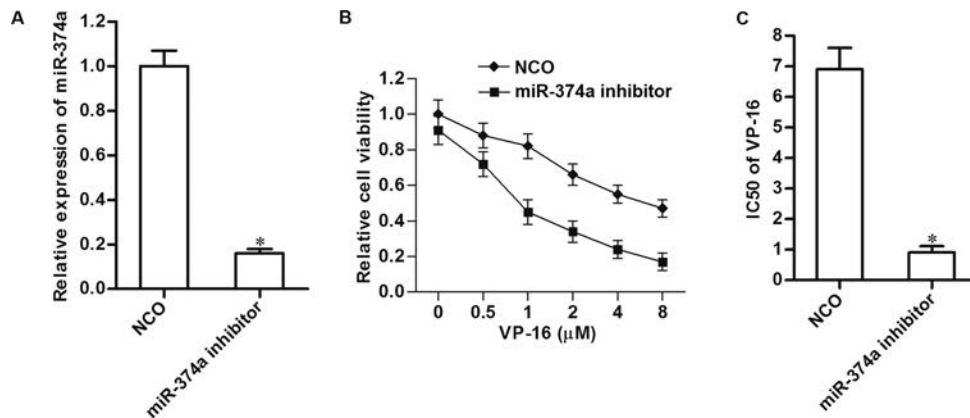
As miR-374a expression was found to be upregulated in glioma cells, we transfected the A172 cells with miR-374a inhibitor to knockdown the miR-374a level (Fig. 2A). Interestingly, we found that miR-374a inhibitor treatment significantly promoted the VP-16-induced cell death in A172 cells (Fig. 2B). The IC<sub>50</sub> of VP-16 to A172 cells was reduced by 85.1% due to the transfection with miR-374a inhibitor (Fig. 2C). These results indicated that adjunctive treatment with miR-374a inhibitor can enhance the cytotoxicity of VP-16 against glioma cells.

### miR-374a Inhibitor Increases the Expression of FOXO1 in Glioma

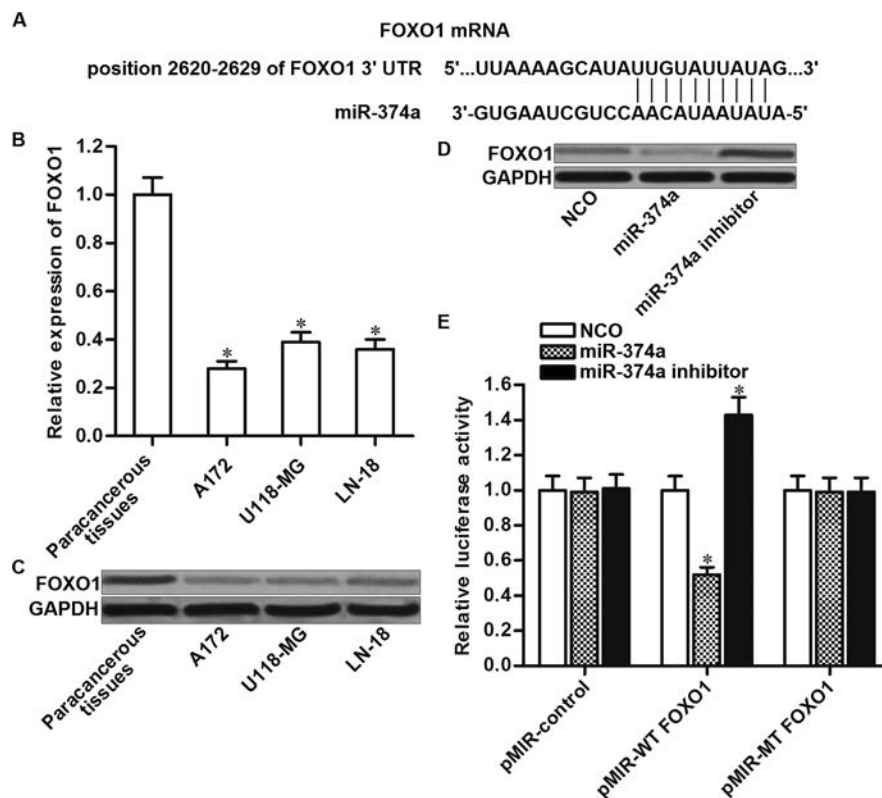
To explore the mechanism by which miR-374a inhibitor enhanced the cytotoxicity of VP-16 against glioma cells, three miRNA databases (TargetScan, miRanda, and PicTar) were used to predict miR-374a binding sites in human mRNA transcripts. These databases showed that FOXO1, a reported tumor suppressor in multiple cancers<sup>19-21</sup>, contains a putative binding site paired with miR-374a (Fig. 3A). Results of qRT-PCR (Fig. 3B) and Western blot analysis (Fig. 3C) showed that glioma cell lines expressed an obviously lower level of FOXO1 compared to the paracancerous cells, in contrast to the upregulation of miR-374a in glioma cells. These results suggested that



**Figure 1.** Overexpression of miR-374a in glioma. (A) Quantitative reverse transcriptase real-time PCR (qRT-PCR) analysis was performed to evaluate the expression profile of miR-374a in glioma patients' tumor tissues and paracancerous tissues ( $n = 25$ ).  $*p < 0.05$  versus paracancerous tissues. (B) Comparison of miR-374a expression between glioma cell lines and paracancerous tissues.  $*p < 0.05$  versus paracancerous tissues.



**Figure 2.** Effect of miR-374a inhibitor adjunctive treatment on etoposide (VP-16)-induced cytotoxicity against glioma. (A) After transfection with miR-374a inhibitor, relative expression level of miR-374a in A172 cells was detected by qRT-PCR analysis. \* $p < 0.05$  versus negative control oligonucleotide (NCO) group. (B) miR-374a inhibitor-transfected A172 cells were treated with different concentrations of VP-16 (0–8  $\mu\text{M}$ ). Cell viability was measured using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assays. (C) Effect of miR-374a inhibitor on changing the  $\text{IC}_{50}$  of VP-16 to A172 was calculated according to the cell viability curve. \* $p < 0.05$  versus NCO group.



**Figure 3.** Effect of miR-374a inhibitor on regulating the forkhead box O 1 (FOXO1) expression in glioma. (A) Putative binding site paired with miR-374a at the 3'-untranslated region (3'-UTR) of FOXO1 mRNA. (B) qRT-PCR analysis was performed to detect the expression of FOXO1 in glioma cell lines and paracancerous cells at the mRNA level. \* $p < 0.05$  versus paracancerous tissues. (C) Protein level of FOXO1 in glioma cell lines and paracancerous cells was analyzed by Western blot assay. (D) Effect of miR-374a (50 pmol/ml) and miR-374a inhibitor (50 pmol/ml) on regulating the expression level of FOXO1 in A172 cells. (E) After cotransfection with miR-374a mimic/inhibitor and pMIR plasmid carrying FOXO1 3'-UTR in A172 cells, luciferase activities were measured using Dual-Luciferase Reporter Assay System according to the manufacturer's instruction. \* $p < 0.05$  versus NCO group.

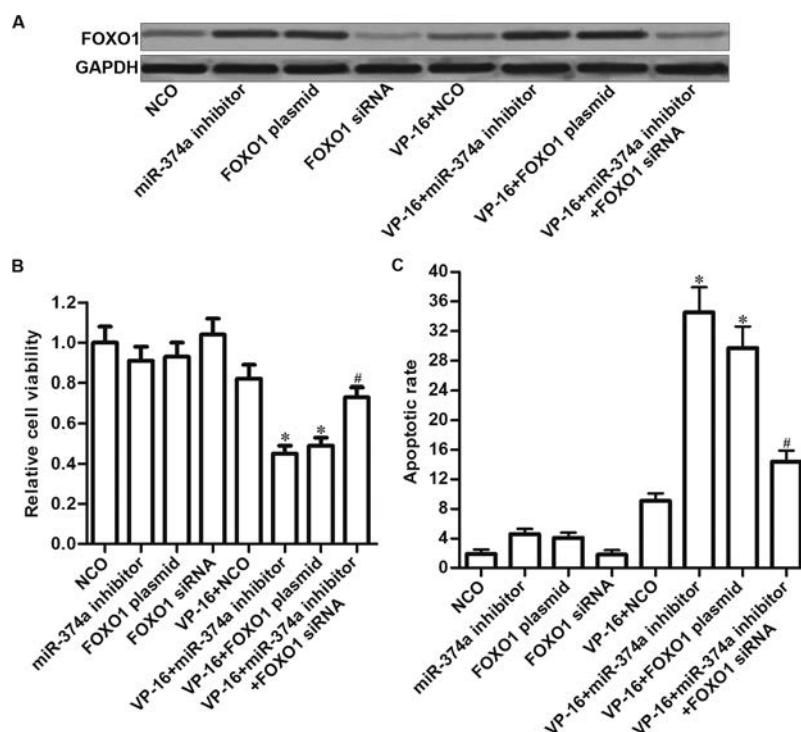


decrease in FOXO1 expression may be induced by over-expression of miR-374a in glioma. We therefore transfected the A172 cells with miR-374a mimic or inhibitor before detection of FOXO1 expression. We found that transfection with miR-374a mimic decreased the protein level of FOXO1, whereas the miR-374a inhibitor treatment was able to increase the expression of FOXO1 (Fig. 3D). To confirm the miR-374a/FOXO1 axis in glioma, we inserted the wild-type or mutant 3'-UTR of FOXO1 into the pMIR reporter followed by performance of dual-luciferase reporter assays. The results showed that miR-374a inhibitor significantly increased the luciferase activities of pMIR reporter containing wild-type FOXO1 3'-UTR but not the mutant pMIR reporter (Fig. 3E). Taken together, we proved that FOXO1 was the target of miR-374a, and miR-374a inhibitor treatment can increase the expression of FOXO1 in glioma.

#### *miR-374a Inhibitor Sensitizes Glioma Cells to VP-16-Induced Apoptosis Through Upregulation of FOXO1*

Since miR-374a inhibitor increased the expression of FOXO1 in glioma, we next investigated whether the

sensitization of miR-374a inhibitor on VP-16-induced cytotoxicity to glioma cells was dependent on the FOXO1 pathway. As shown in Figure 4A, transfection with FOXO1 eukaryotic expression vector obviously over-expressed FOXO1 in A172 cells. FOXO1 siRNA was found to abolish the effect of miR-374a inhibitor on increasing FOXO1 expression. Results of cell viability assays showed that the FOXO1 siRNA partially “rescued” the A172 cells cotreated with VP-16 and miR-374a inhibitor. On the contrary, enforced expression of FOXO1 directly through the FOXO1 plasmid transfection was observed to increase the cytotoxicity of VP-16 against A172 cells, similar to the miR-374a inhibitor (Fig. 4B). Furthermore, the results of flow cytometry showed that FOXO1 plasmid transfection enhanced the VP-16-induced apoptosis, similar to the miR-374a inhibitor. However, knockdown of FOXO1 using its specific siRNA was found to inhibit the miR-374a inhibitor-promoted apoptosis induced by VP-16 in A172 cells (Fig. 4C). Taken together, we demonstrated that miR-374a inhibitor sensitizes glioma cells to VP-16-induced apoptosis through upregulation of FOXO1.



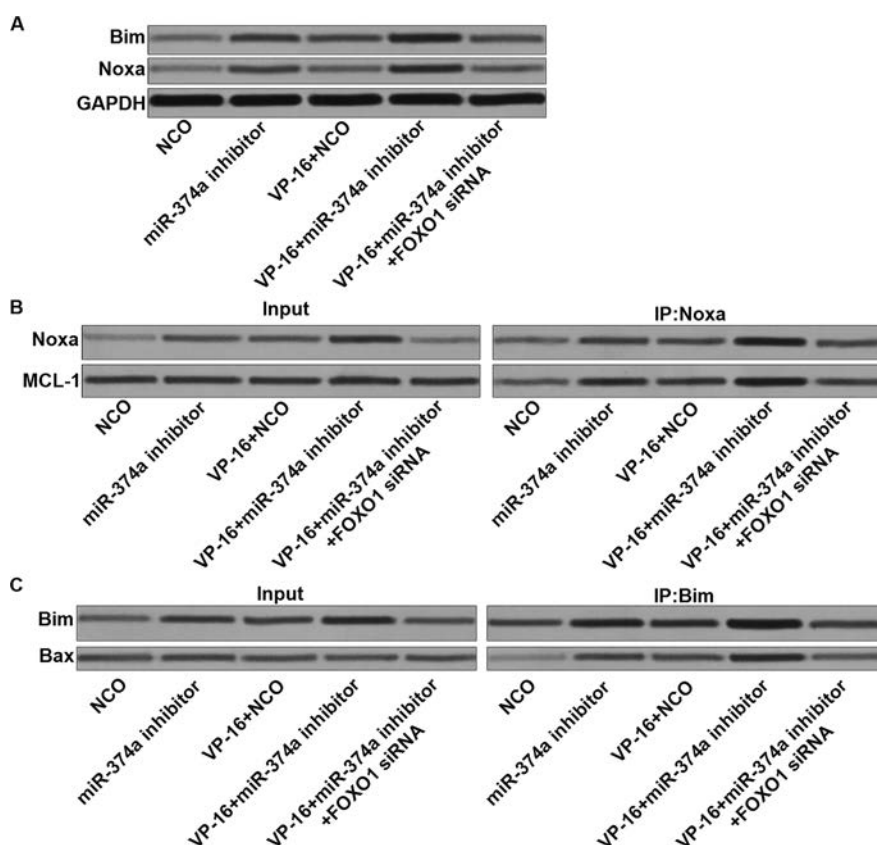
**Figure 4.** miR-374a inhibitor sensitizes glioma cells to VP-16-induced apoptosis through upregulation of FOXO1. (A) Expression level of FOXO1 in A172 cells was detected using Western blot analysis after treatment with miR-374a inhibitor, VP-16 (1  $\mu$ M), FOXO1 siRNA, and FOXO1 plasmid. (B) Cell viability of A172 was detected using MTT assays after treatment with miR-374a inhibitor, VP-16 (1  $\mu$ M), FOXO1 siRNA, and FOXO1 plasmid. \* $p$ <0.05 versus VP-16+NCO group. # $p$ <0.05 versus VP-16+miR-374a inhibitor group. (C) Cell apoptotic rate of A172 was detected using flow cytometry after treatment with miR-374a inhibitor, VP-16 (1  $\mu$ M), FOXO1 siRNA, and FOXO1 plasmid. \* $p$ <0.05 versus VP-16+NCO group. # $p$ <0.05 versus VP-16+miR-374a inhibitor group.

### miR-374a Inhibitor Promotes Bim and Noxa Expression in VP-16-Treated Glioma Cells

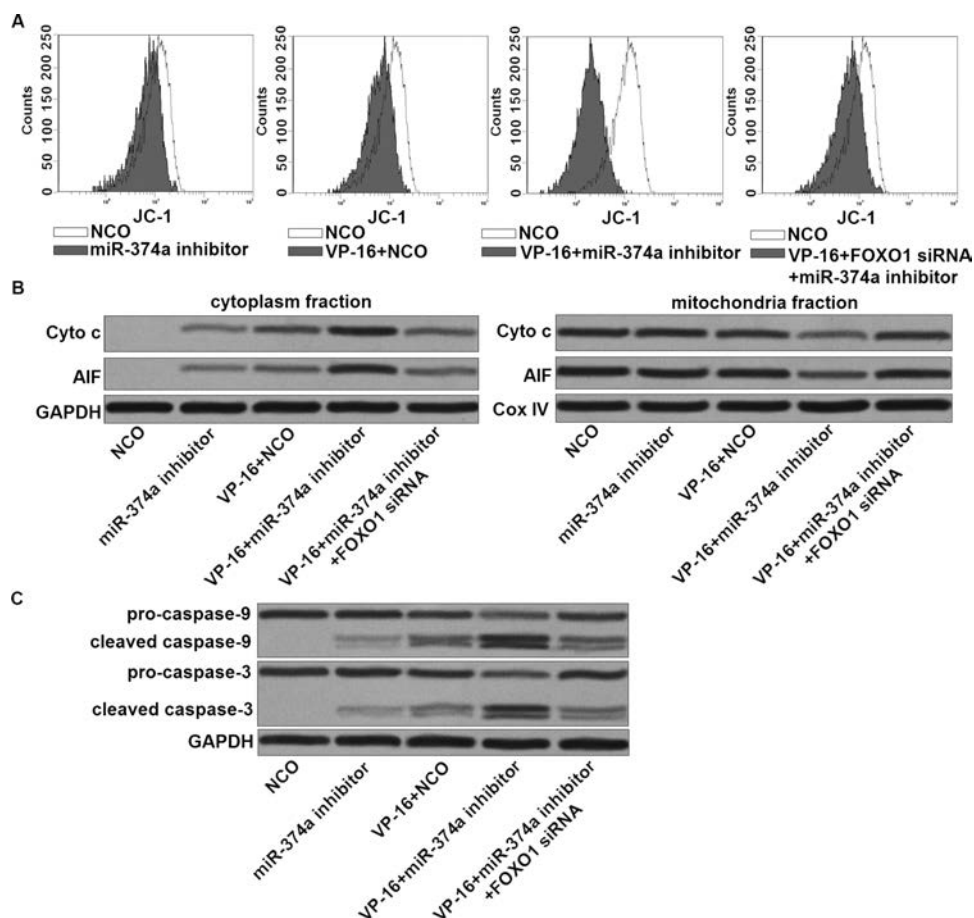
Bim and Noxa expression is directly transactivated by FOXO1<sup>22,23</sup>. We therefore investigated the effect of miR-374a inhibitor on regulating the Bim and Noxa expression in VP-16-treated A172 cells. As shown in Figure 5A, cotreatment with miR-374a inhibitor obviously promoted the expression level of Bim and Noxa in VP-16-treated A172 cells, whereas FOXO1 siRNA inhibited the effect of miR-374a inhibitor. Results of coimmunoprecipitation showed that miR-374a inhibitor promoted interaction with Noxa and MCL-1 (Fig. 5B). In addition, interaction with Bim and Bax also could be enhanced due to the miR-374a inhibitor transfection in VP-16-treated A172 cells (Fig. 5C). However, cotransfection with FOXO1 siRNA was found to inhibit the activities of Bim and Noxa in miR-374a and VP-16 cotreated A172 cells. Taken together, we demonstrated that miR-374a inhibitor increased the expression of FOXO1, and thus promoted the function of Bim and Noxa in VP-16-treated glioma cells.

### miR-374a Inhibitor Promotes Mitochondrial Apoptosis in VP-16-Treated Glioma Cells

Since miR-374a inhibitor/FOXO1 axis enhanced the function of Bim and Noxa, which act as the key proapoptotic proteins in the mitochondrial apoptosis pathway<sup>24,25</sup>, we next investigated the effect of miR-374a inhibitor on mitochondrial apoptosis in VP-16-treated glioma cells. We found that adjunctive treatment with miR-374a inhibitor obviously expanded the decrease in mitochondrial membrane potential (MMP) in the VP-16-treated A172 cells. However, the effect of miR-374a inhibitor can be suppressed through knockdown of FOXO1 (Fig. 6A). As the downstream of the mitochondria collapses, cotreatment with miR-374a inhibitor and VP-16 induced drastic release of cyto c and AIF, which are the apoptotic inducers<sup>26</sup> from the mitochondria into the cytoplasm (Fig. 6B). In the presence of these apoptotic inducers, caspase 9 and caspase 3, which act as apoptosis executors, were triggered in the VP-16 and miR-374a-cotreated A172 cells (Fig. 6C). These data demonstrated



**Figure 5.** Effect of miR-374a inhibitor on regulating the expression of Bim and Noxa in VP-16-induced glioma cells. (A) Expression level of Bim and Noxa in A172 cells was detected using Western blot analysis after treatment with miR-374a inhibitor, VP-16 (1  $\mu$ M), and FOXO1 siRNA. (B) Interaction with Noxa and MCL-1 in A172 cells was evaluated using coimmunoprecipitation assay after treatment with miR-374a inhibitor, VP-16 (1  $\mu$ M), and FOXO1 siRNA. (C) Interaction with Bim and Bax in A172 cells was evaluated using coimmunoprecipitation assay after treatment with miR-374a inhibitor, VP-16 (1  $\mu$ M), and FOXO1 siRNA.



**Figure 6.** miR-374a/FOXO1 axis promotes mitochondrial apoptosis in VP-16-treated glioma cells. (A) Mitochondrial membrane potential of A172 cells was measured using flow cytometry after treatment with miR-374a inhibitor, VP-16 (1  $\mu$ M), and FOXO1 siRNA. (B) Release of cyto c and AIF from mitochondria into cytoplasm in A172 cells was evaluated using Western blot after treatment with miR-374a inhibitor, VP-16 (1  $\mu$ M), and FOXO1 siRNA. (C) Cleavage of caspase 9 and caspase 3 in A172 cells was detected using Western blot after treatment with miR-374a inhibitor, VP-16 (1  $\mu$ M), and FOXO1 siRNA.

that miR-374a inhibitor can promote mitochondrial apoptosis in VP-16-treated glioma cells through the upregulation of FOXO1 expression.

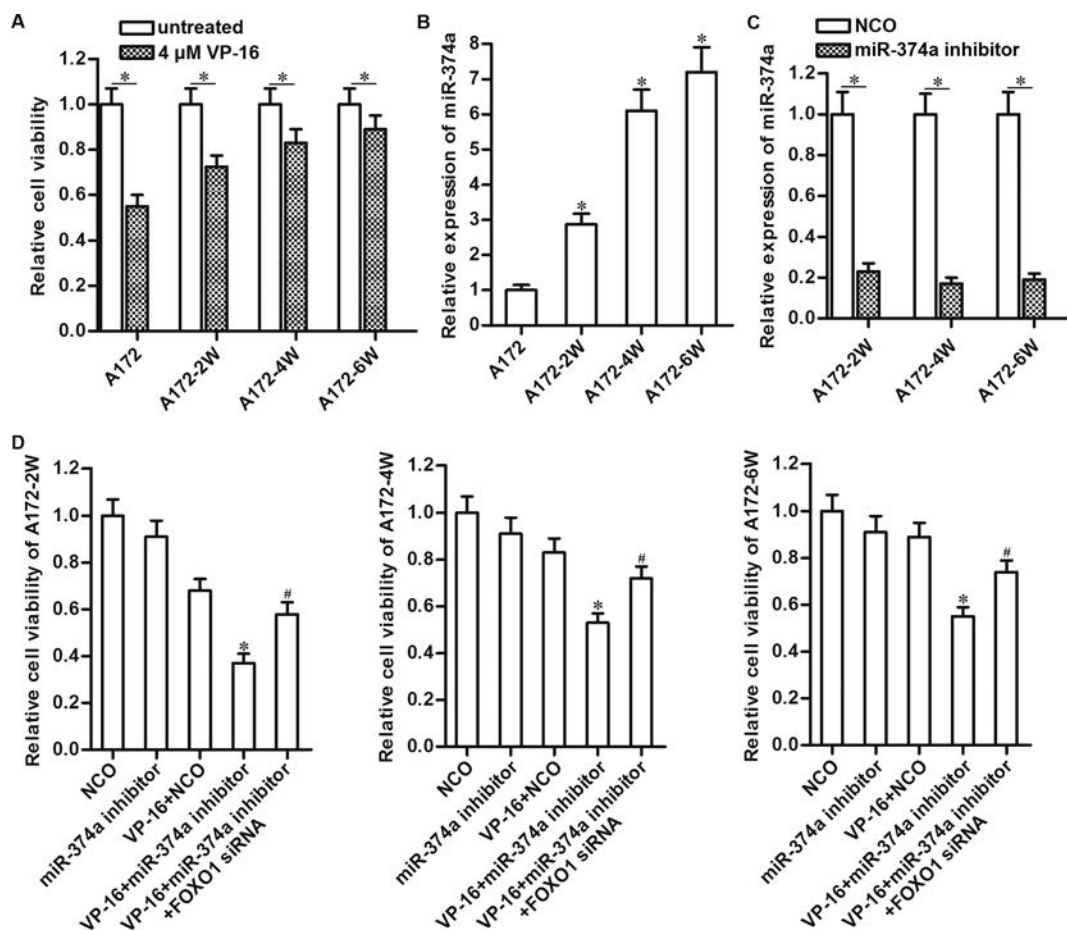
#### *miR-374a Inhibitor Attenuates the Required Resistance of VP-16 in Glioma Cells*

Under the treatment with equal concentration of VP-16 (4  $\mu$ M), we found that these A172-2W, A172-4W, and A172-6W cells showed increasing resistance against VP-16 (Fig. 7A). Interestingly, we found that these VP-16 pretreated A172 cells exhibited obviously higher levels of miR-374a compared to the A172 cells (Fig. 7B). Next, we transfected these VP-16-resistant cells with miR-374a inhibitor (the transfection efficiency of miR-374a inhibitor is shown in Fig. 7C). As the results, knockdown of miR-374a was found to increase the sensitivity of A172-2W, A172-4W, and A172-6W cells to VP-16 (4  $\mu$ M) treatment. However, FOXO1 siRNA was found to partially “rescue” the A172-2W, A172-4W, and A172-6W cells cotreated with

VP-16 and miR-374a inhibitor (Fig. 7D). Taken together, we demonstrated that miR-374a inhibitor can attenuate the required resistance of VP-16 in glioma cells through upregulation of FOXO1 expression.

## DISCUSSION

Previous studies have indicated that formation of chemoresistance is usually accompanied with dysregulation of miRNAs in cancers<sup>27,28</sup>. Among these dysregulated miRNAs, miR-374a is usually overexpressed in several cancers. Moreover, the upregulation of miR-374a in cancers is found to be responsible for higher proliferation and metastasis<sup>29-31</sup>. Overexpression of miR-374a is reported to decrease the sensitivity of cisplatin to ovarian cancer cells<sup>32</sup>. Therefore, miR-374a may represent a key target for cancer therapy. In this study, we observed a significant increase in miR-374a expression in glioma tissues and cell lines. Interestingly, we found that



**Figure 7.** miR-374a inhibitor attenuates the required resistance of VP-16 in glioma cells through upregulation of FOXO1 expression. (A) Cell viability of A172, A172-2W, A172-4W, and A172-6W was detected using MTT assays after treatment with 4  $\mu$ M VP-16. \* $p$ <0.05. (B) Expression level of miR-374a in A172, A172-2W, A172-4W, and A172-6W cells. \* $p$ <0.05 versus A172 cells. (C) Transfection efficiency of miR-374a inhibitor in A172-2W, A172-4W, and A172-6W was evaluated by qRT-PCR analysis. \* $p$ <0.05. (D) Cell viability of A172-2W, A172-4W, and A172-6W was detected using MTT assays after treatment with miR-374a inhibitor, VP-16 (4  $\mu$ M), and FOXO1 siRNA. \* $p$ <0.05 versus VP-16+NCO group. # $p$ <0.05 versus VP-16 + miR-374a inhibitor group.

inhibition of miR-374a by using its specific antisense oligonucleotide can increase the sensitivity of glioma cells to VP-16 treatment. We demonstrated that combination with miR-374a inhibitor can be considered as a potential approach to sensitize the VP-16-induced cytotoxicity against glioma.

FOXO1 is a cellular transcription factor that regulates expression of some important genes. In cancer cells, FOXO1 acts as a tumor suppressor by regulating DNA repair and cell cycle transition<sup>33-35</sup>. FOXO1 facilitates cell apoptosis because FOXO1 promotes transcriptional activities of Bim and Noxa, which are the key proapoptotic proteins in the mitochondrial apoptosis pathway<sup>24,25</sup>. Previous studies have indicated that absence of FOXO1 induces tumorigenesis, cancer development, and chemoresistance<sup>9,36</sup>. Therefore, FOXO1 may be a potential target for improving cancer therapy.

In the present study, we observed significant downregulation of FOXO1 in glioma cells. However, we found that transfection with miR-374a inhibitor can restore the FOXO1 expression in glioma cells. Furthermore, we showed that the promotion of miR-374a inhibitor on VP-16-induced cytotoxicity against glioma was dependent on the upregulation of FOXO1 expression. In the pathway of apoptosis induced by VP-16 and miR-374a-cotreatment, we observed an obvious overexpression of Bim and Noxa, which are the key mediators of mitochondrial apoptosis. As a result, we found drastic mitochondria collapse followed by release of cyto c and AIF in these VP-16 and miR-374a-cotreated gliomas.

In conclusion, we demonstrated the effect of miR-374a inhibitor/FOXO1 axis on sensitizing glioma cells to VP-16-based chemotherapy. However, whether the strategy of miR-374a knockdown can be used in the clinic is required to be tested in the future.



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## REFERENCES

- Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. *CA Cancer J Clin.* 2013;63(1):11–30.
- van den Bent MJ. Chemotherapy for low-grade glioma: When, for whom, which regimen? *Curr Opin Neurol.* 2015;28(6):633–8.
- Stewart DJ. The role of chemotherapy in the treatment of gliomas in adults. *Cancer Treat Rev.* 1989;16(3):129–60.
- Sawada M, Nakashima S, Banno Y, Yamakawa H, Hayashi K, Takenaka K, Nishimura Y, Sakai N, Nozawa Y. Ordering of ceramide formation, caspase activation, and Bax/Bcl-2 expression during etoposide-induced apoptosis in C6 glioma cells. *Cell Death Differ.* 2000;7(9):761–72.
- Wang F, Bhat K, Doucette M, Zhou S, Gu Y, Law B, Liu X, Wong ET, Kang JX, Hsieh TC, Qian SY, Wu E. Docosahexaenoic acid (DHA) sensitizes brain tumor cells to etoposide-induced apoptosis. *Curr Mol Med.* 2011;11(6):503–11.
- Matsumoto Y, Tamiya T, Nagao S. Resistance to topoisomerase II inhibitors in human glioma cell lines overexpressing multidrug resistant associated protein (MRP) 2. *J Med Invest.* 2005;52(1–2):41–8.
- Benyahia B, Huguet S, Declèves X, Mokhtari K, Crinière E, Bernaudin JF, Scherrmann JM, Delattre JY. Multidrug resistance-associated protein MRP1 expression in human gliomas: Chemosensitization to vincristine and etoposide by indomethacin in human glioma cell lines overexpressing MRP1. *J Neurooncol.* 2004;66(1–2):65–70.
- Zhao J, Cao J, Zhou L, Du Y, Zhang X, Yang B, Gao Y, Wang Y, Ma N, Yang W. MiR-1260b inhibitor enhances the chemosensitivity of colorectal cancer cells to fluorouracil by targeting PDCD4/IGF1. *Oncol Lett.* 2018;16(4):5131–9.
- Qin Y, Li L, Wang F, Zhou X, Liu Y, Yin Y, Qi X. Knockdown of mir-135b sensitizes colorectal cancer cells to oxaliplatin-induced apoptosis through increase of FOXO1. *Cell Physiol Biochem.* 2018;48(4):1628–37.
- Bartel DP. MicroRNAs: Target recognition and regulatory functions. *Cell* 2009;136(2):215–33.
- Iorio MV, Croce CM. microRNA involvement in human cancer. *Carcinogenesis* 2012;33(6):1126–33.
- Di Leva G, Garofalo M, Croce CM. MicroRNAs in cancer. *Annu Rev Pathol.* 2014;9:287–314.
- Liu J, Tang Q, Li S, Yang X. Inhibition of HAX-1 by miR-125a reverses cisplatin resistance in laryngeal cancer stem cells. *Oncotarget* 2016;7(52):86446–56.
- He H, Tian W, Chen H, Deng Y. MicroRNA-101 sensitizes hepatocellular carcinoma cells to doxorubicin-induced apoptosis via targeting Mcl-1. *Mol Med Rep.* 2016;13(2):1923–9.
- Wang N, Zhu M, Tsao SW, Man K, Zhang Z, Feng Y. MiR-23a-mediated inhibition of topoisomerase I expression potentiates cell response to etoposide in human hepatocellular carcinoma. *Mol Cancer* 2013;12(1):119.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>-</sup>(Delta Delta C(T)) method. *Methods* 2001;25(4):402–8.
- Wang J, Tian X, Han R, Zhang X, Wang X, Shen H, Xue L, Liu Y, Yan X, Shen J, Mannoor K, Deepak J, Donahue JM, Stass SA, Xing L, Jiang F. Downregulation of miR-486-5p contributes to tumor progression and metastasis by targeting protumorigenic ARHGAP5 in lung cancer. *Oncogene* 2014;33(9):1181–9.
- Prathapan A, Vineetha VP, Raghu KG. Protective effect of Boerhaavia diffusa L. against mitochondrial dysfunction in angiotensin II induced hypertrophy in H9c2 cardiomyoblast cells. *PLoS One* 2014;9(4):e96220.
- Zhang B, Gui LS, Zhao XL, Zhu LL, Li QW. FOXO1 is a tumor suppressor in cervical cancer. *Genet Mol Res.* 2015;14(2):6605–16.
- Zhang L, Quan H, Wang S, Li X, Che X. MiR-183 promotes growth of non-small cell lung cancer cells through FoxO1 inhibition. *Tumour Biol.* 2015;36(10):8121–6.
- Yu JJ, Wu YX, Zhao FJ, Xia SJ. miR-96 promotes cell proliferation and clonogenicity by down-regulating of FOXO1 in prostate cancer cells. *Med Oncol.* 2014;31(4):910.
- Chi HC, Chen SL, Cheng YH, Lin TK, Tsai CY, Tsai MM, Lin YH, Huang YH, Lin KH. Chemotherapy resistance and metastasis-promoting effects of thyroid hormone in hepatocarcinoma cells are mediated by suppression of FoxO1 and Bim pathway. *Cell Death Dis.* 2016;7(8):e2324.
- Valis K, Prochazka L, Boura E, Chladova J, Obsil T, Rohlena J, Truksa J, Dong LF, Ralph SJ, Neuzil J. Hippo/Mst1 stimulates transcription of the proapoptotic mediator NOXA in a FoxO1-dependent manner. *Cancer Res.* 2011;71(3):946–54.
- Han J, Goldstein LA, Hou W, Rabinowich H. Functional linkage between NOXA and Bim in mitochondrial apoptotic events. *J Biol Chem.* 2007;282(22):16223–31.
- Obexer P, Geiger K, Ambros PF, Meister B, Ausserlechner MJ. FKHRL1-mediated expression of Noxa and Bim induces apoptosis via the mitochondria in neuroblastoma cells. *Cell Death Differ.* 2007;14(3):534–47.
- Kim BM, Choi YJ, Lee YH, Joe YA, Hong SH. N,N-Dimethyl phytosphingosine sensitizes HL-60/MX2, a multidrug-resistant variant of HL-60 cells, to doxorubicin-induced cytotoxicity through ROS-mediated release of cytochrome c and AIF. *Apoptosis* 2010;15(8):982–93.
- Ou Y, Zhai D, Wu N, Li X. Downregulation of miR-363 increases drug resistance in cisplatin-treated HepG2 by dysregulating Mcl-1. *Gene* 2015;572(1):116–22.
- Zarogoulidis P, Petanidis S, Kioseoglou E, Domvri K, Anastakis D, Zarogoulidis K. MiR-205 and miR-218 expression is associated with carboplatin chemoresistance and regulation of apoptosis via Mcl-1 and Survivin in lung cancer cells. *Cell Signal.* 2015;27(8):1576–88.
- Xu X, Wang W, Su N, Zhu X, Yao J, Gao W, Hu Z, Sun Y. miR-374a promotes cell proliferation, migration and invasion by targeting SRCIN1 in gastric cancer. *FEBS Lett.* 2015;589(3):407–13.
- Cai J, Guan H, Fang L, Yang Y, Zhu X, Yuan J, Wu J, Li M. MicroRNA-374a activates Wnt/β-catenin signaling to promote breast cancer metastasis. *J Clin Invest.* 2013;123(2):566–79.
- Wang Y, Xin H, Han Z, Sun H, Gao N, Yu H. MicroRNA-374a promotes esophageal cancer cell proliferation via Axin2 suppression. *Oncol Rep.* 2015;34(4):1988–94.
- Li N, Yang L, Wang H, Yi T, Jia X, Chen C, Xu P. MiR-130a and miR-374a function as novel regulators of cisplatin

- resistance in human ovarian cancer A2780 cells. *PLoS One* 2015;10(6):e0128886.
33. Greer EL, Brunet A. FOXO transcription factors at the interface between longevity and tumor suppression. *Oncogene* 2005;24(50):7410–25.
  34. Goto T, Takano M, Hirata J, Tsuda H. The involvement of FOXO1 in cytotoxic stress and drug-resistance induced by paclitaxel in ovarian cancers. *Br J Cancer* 2008;98(6):1068–75.
  35. Zhao Z, Qin L, Li S. miR-411 contributes the cell proliferation of lung cancer by targeting FOXO1. *Tumour Biol.* 2016;37(4):5551–60.
  36. Lang C, Xu M, Zhao Z, Chen J, Zhang L. MicroRNA-96 expression induced by low-dose cisplatin or doxorubicin regulates chemosensitivity, cell death and proliferation in gastric cancer SGC7901 cells by targeting FOXO1. *Oncol Lett.* 2018;16(3):4020–6.