

## Original Article

# Formononetin sensitizes glioma cells to doxorubicin through preventing EMT via inhibition of histone deacetylase 5

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Received February 6, 2015; Accepted March 30, 2015; Epub June 1, 2015; Published June 15, 2015

**Abstract:** Chemoresistance is a major obstacle to successful chemotherapy for glioma. Formononetin is a novel herbal isoflavonoid isolated from *Astragalus membranaceus* and possesses antitumorogenic properties. In the present study, we investigated the anti-proliferative effects of formononetin on human glioma cells, and further elucidated the molecular mechanism underlying the anti-tumor property. We found that formononetin enhanced doxorubicin cytotoxicity in glioma cells. Combined treatment with formononetin reversed the doxorubicin-induced epithelial-mesenchymal transition (EMT) in tumor cells. Moreover, we found that formononetin treatment significantly decreased the expression of HDAC5. Overexpression of HDAC5 diminished the suppressive effects of formononetin on glioma cell viability. Furthermore, knockdown of HDAC5 by siRNA inhibited the doxorubicin-induced EMT in glioma cells. Taken together, these results demonstrated that formononetin-combined therapy may enhance the therapeutic efficacy of doxorubicin in glioma cells by preventing EMT through inhibition of HDAC5.

**Keywords:** Glioma, epithelial-mesenchymal transition, HDAC5, combination treatment

## Introduction

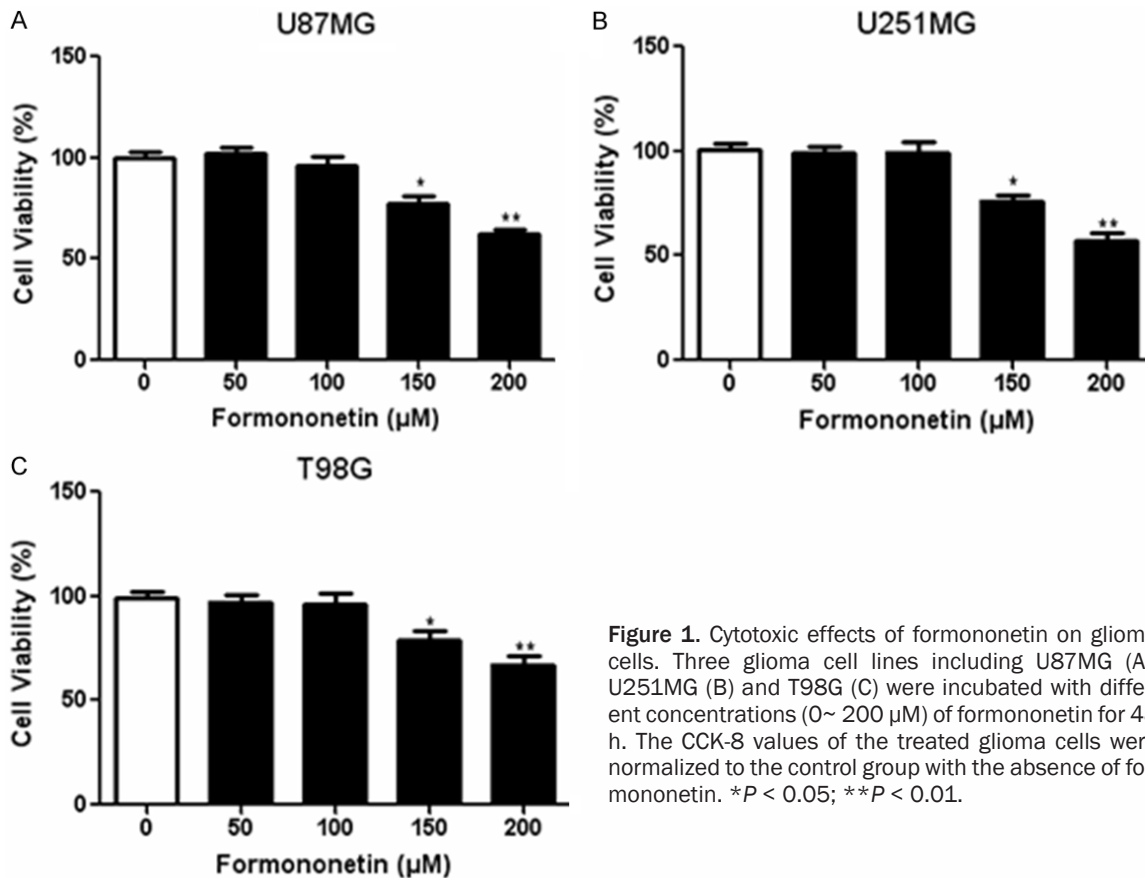
Glioblastoma multiforme (GBM) is the most common primary brain tumor, which is characterized by high aggressiveness and poor prognosis [1, 2]. The lethality of this malignancy is mainly due to the abnormal proliferation and invasiveness of glioma cells. The current strategy for the treatment of GBM is general palliative treatment, including surgical palliative resection, standard chemotherapy, and focal radiotherapy [3-5]. However, the development of acquired drug resistance to conventional chemotherapeutics has become a major obstacle in GBM treatment [6]. Such limitation highlights the imperative need for identifying novel treatment strategies which may help overcome drug resistance and enhance tumor cell response to anti-cancer drugs.

It has been acknowledged that the pathogenesis of glioma is a multistep process regulated by aberrantly protein expression and altera-

tions of morphological and molecular features during malignant progression [7, 8]. Epithelial-mesenchymal transition (EMT) is a complex, reversible process which induces epithelial cells to transform to mesenchymal phenotype [9, 10]. Accumulating evidences suggest that EMT plays an important role in regulating the chemoresistance properties of glioma [11].

Traditional Chinese herbs are significant sources of drugs that serve as potential therapeutic compounds for cancer treatment. *Astragalus membranaceus* (*Radix Astragali*) has a long history of medicinal use in traditional Chinese medicine as an immunomodulating agent to treat diarrhea, anorexia and fatigue [12]. Formononetin, an active component isolated from *Astragalus membranaceus*, possesses diverse pharmacological benefits such as anti-inflammatory and immuno-modulatory activity [13]. Accumulating evidences also demonstrated the anticancer activity of formononetin on several cancer types such as breast cancer,

## Formononetin sensitizes glioma cells via HDAC5



**Figure 1.** Cytotoxic effects of formononetin on glioma cells. Three glioma cell lines including U87MG (A), U251MG (B) and T98G (C) were incubated with different concentrations (0~ 200 μM) of formononetin for 48 h. The CCK-8 values of the treated glioma cells were normalized to the control group with the absence of formononetin. \* $P < 0.05$ ; \*\* $P < 0.01$ .

prostate cancer and cervical cancer [14-16]. However, the effect of formononetin on human glioma has not been elucidated. Thus, the present study aimed to explore the anti-proliferative effects of formononetin on glioma cells, and further elucidate the molecular mechanism underlying the anti-tumor property on human glioma.

### Materials and methods

#### Cell culture and reagents

Human glioma cell lines U87MG, U251MG, and T98G were purchased from the ATCC (Manassas, VA, USA) and cultured in DMEM (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were maintained at 37°C in 5% CO<sub>2</sub> incubator. Formononetin (purity > 99%) and doxorubicin (Dox) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The HDAC5 plasmid, HDAC5 siRNA and negative control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

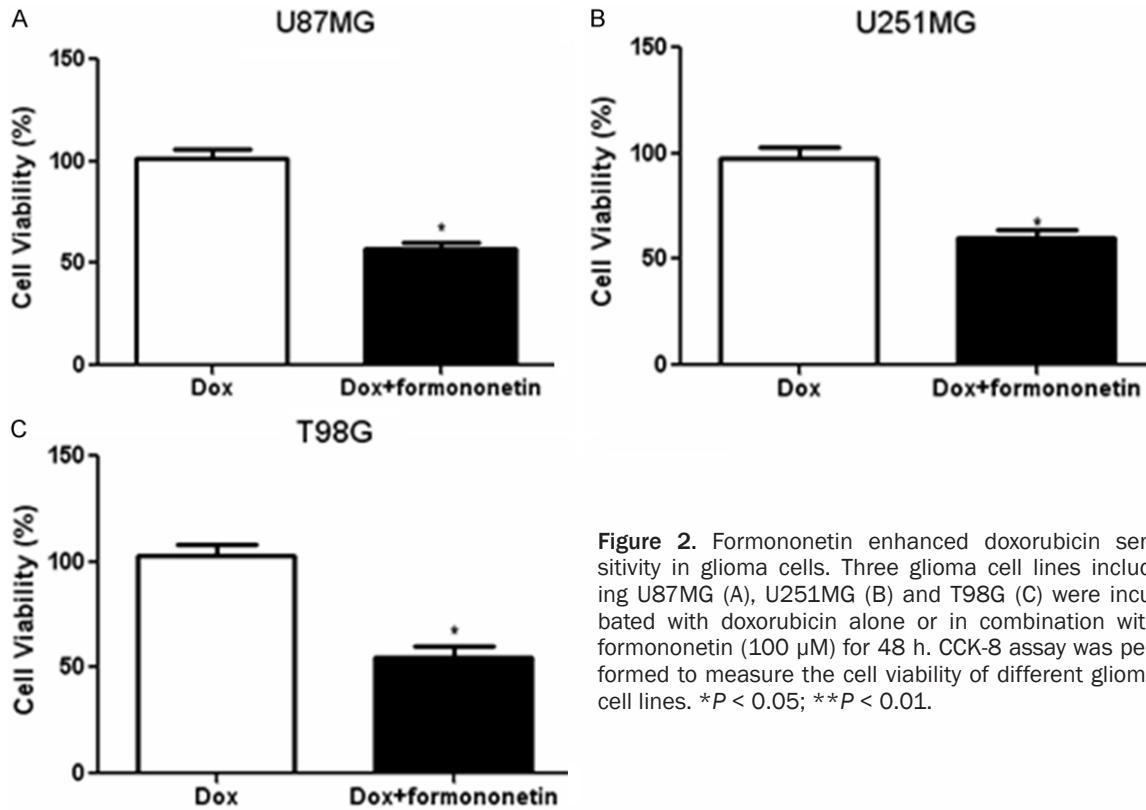
#### CCK-8 assay

Tumor cells were seeded onto 96-well plates at 3000 cells/well. The medium was replaced with the corresponding serum-free medium for 24 h to synchronize the cell cycle, and then serum-free medium was replaced with complete medium containing the drugs at the indicated concentrations for 48 h. Then 10 μL/well CCK8 solution (Dojindo, Kumamoto, Japan) was added, the plates incubated for 3 h, and absorbance was measured at 450 nm using an MRX II microplate reader (Dynex, Chantilly, VA, USA).

#### Transfection

Cells were transfected with HDAC5 plasmid, HDAC5 siRNA or negative control siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The transfection medium (Opti-MEM; Gibco) was replaced with complete medium 12 h after transfection, and the cells were incubated for the indicated times.

## Formononetin sensitizes glioma cells via HDAC5



**Figure 2.** Formononetin enhanced doxorubicin sensitivity in glioma cells. Three glioma cell lines including U87MG (A), U251MG (B) and T98G (C) were incubated with doxorubicin alone or in combination with formononetin (100  $\mu$ M) for 48 h. CCK-8 assay was performed to measure the cell viability of different glioma cell lines. \* $P < 0.05$ ; \*\* $P < 0.01$ .

### Western blot analysis

Cells were lysed in 50  $\mu$ l cell lysis buffer (Cell Signaling, Danvers, MA, USA) containing protease inhibitors (Sigma). The protein concentration was quantified using the BCA Protein Kit (Thermo, Rockford, IL, USA). Cell lysates were separated by 10% SDS-PAGE and proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were then incubated with primary antibodies (E-cadherin, Vimentin or HDAC5, diluted 1:1000; Abcam, Cambridge, USA) at 4°C overnight. The membranes were washed three times with TBS/T and then incubated with the appropriate HRP-conjugated secondary antibodies for 1 h at room temperature. Protein expression was detected by chemiluminescence (GE Healthcare, Piscataway, NJ, USA).

### Statistical analysis

Each experiment was performed in triplicate, and repeated at least three times. All the data were presented as means  $\pm$  SD and treated for statistics analysis by SPSS program. Comparison between groups was made using AN-

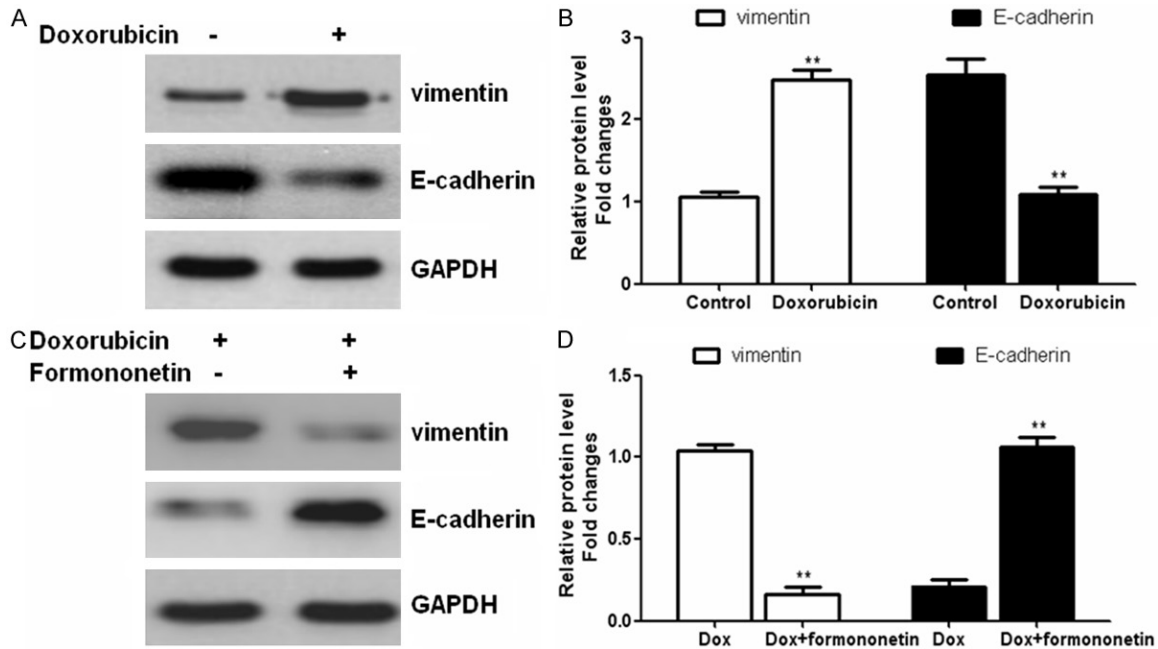
OVA and statistically significant difference was defined as  $P < 0.05$ .

### Results

#### *Formononetin enhanced doxorubicin sensitivity in glioma cells*

Firstly, CCK-8 assay was performed to determine the appropriate concentration of formononetin for combined treatment with doxorubicin. A series of formononetin concentrations ranging from 0~ 200  $\mu$ M were incubated with three glioma cells lines (U87MG, U251MG and T98G) and data from CCK-8 assay showed that formononetin exerted little cytotoxicity in cancer cells between 0 and 100  $\mu$ M. However, higher concentrations of formononetin (150, 200  $\mu$ M) significantly inhibited the viability of the three cell lines (**Figure 1A-C**). Therefore, 100  $\mu$ M formononetin was used for further co-administration with doxorubicin. To evaluate the synergistic cytotoxic effects of doxorubicin combined with formononetin, we used CCK-8 assay to measure cell viability treated with doxorubicin alone or in combination with formononetin for 48 h. At a result, the doxorubicin sensitivity was increased in U87MG, U251MG

## Formononetin sensitizes glioma cells via HDAC5



**Figure 3.** Formononetin treatment altered the expression of doxorubicin-induced EMT-markers. U87MG cells were incubated with doxorubicin alone (A and B) or in combination with Formononetin (100  $\mu$ M) (C and D) for 48 h. Western blot was performed to determine the expression of EMT markers E-cadherin and vimentin. \* $P < 0.05$ ; \*\* $P < 0.01$ .

and T98G cells after co-administration with formononetin (Figure 2A-C). These results suggested that formononetin could enhance the sensitivity of doxorubicin in glioma cells.

### Formononetin inhibited doxorubicin-induced EMT

In order to investigate whether doxorubicin can induce EMT in tumor cells, we evaluated the expression of epithelial/mesenchymal markers in glioma cells treated with doxorubicin for 48 h. Results showed that administration of doxorubicin significantly enhanced the expression of vimentin and decreased the expression of E-cadherin in U87MG cells (Figure 3A and 3B). However, combine treatment with formononetin decreased the expression of vimentin and increased the E-cadherin levels, indicating that formononetin reversed the doxorubicin-induced EMT in glioma cells (Figure 3C, 3D).

### Formononetin treatment suppressed the expression of HDAC5

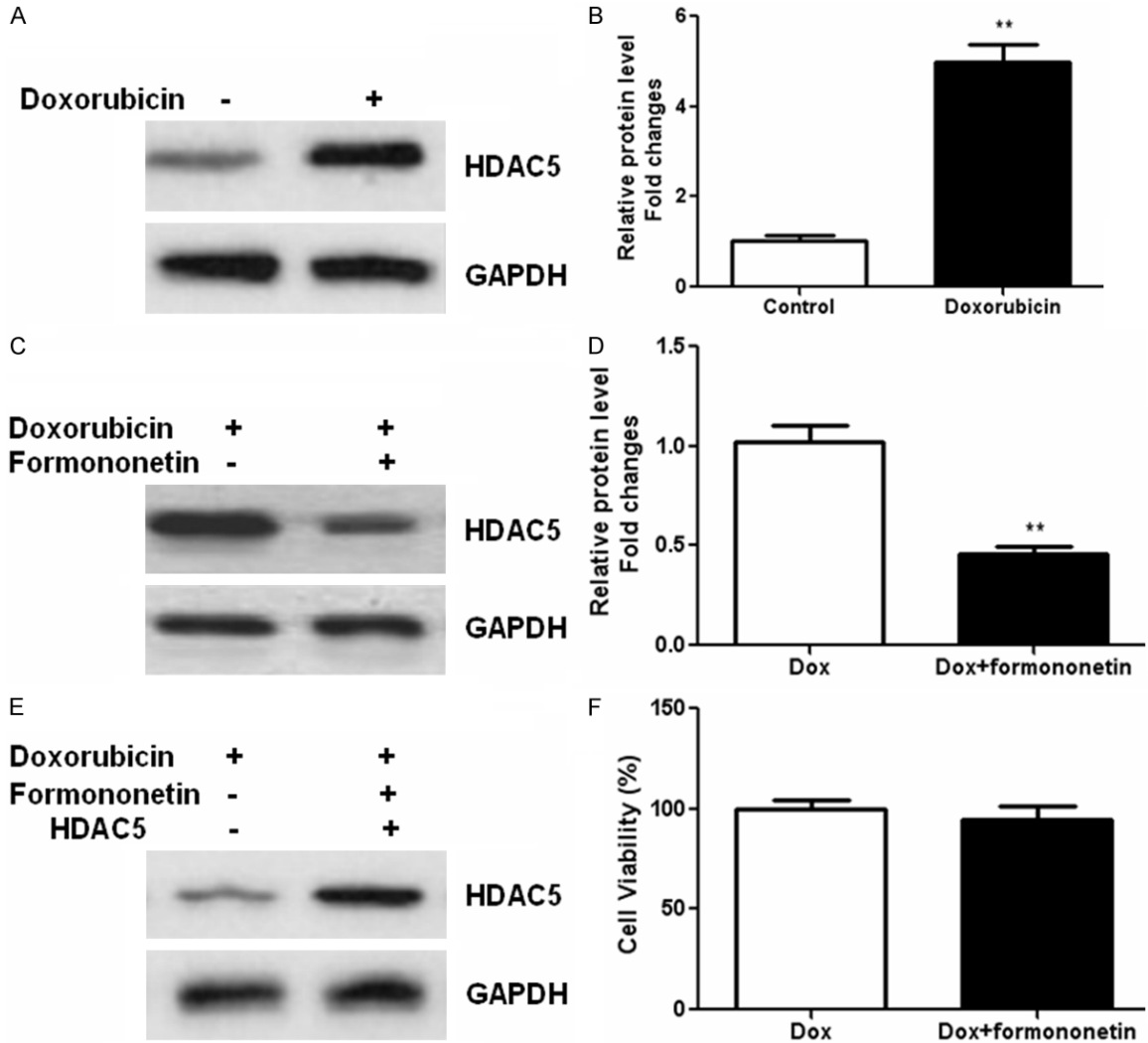
Our previous study showed that HDAC5 promoted glioma cell proliferation [17]; we further examined the relevance of HDAC5 with chemo-

resistance in tumor cells. We found that HDAC5 was significantly increased in doxorubicin-treated glioma cells (Figure 4A and 4B). However, formononetin co-treatment reduced the expression of HDAC5 in glioma cells (Figure 4C and 4D). We then transfected U87MG cells with the plasmid encoding HDAC5 (Figure 4E). As a result, overexpression of HDAC5 diminished the suppressive effects of formononetin on glioma cell viability (Figure 4F). These data implied that formononetin sensitized glioma cells through inhibition of HDAC5.

### Knockdown of HDAC5 diminished the doxorubicin-induced EMT

Nest we investigated whether HDAC5 regulated doxorubicin-induced EMT, RNAi was applied to knockdown the expression of HDAC5 in glioma cells. The HDAC5 siRNA-transfected glioma cells were incubated with doxorubicin alone or in combination with formononetin for 48 h. CCK-8 assay revealed that the cell viability of formononetin plus doxorubicin-treated cells was not significantly different compared to the doxorubicin-treated cells transfected with HDAC5 siRNA (Figure 5A-C), suggesting that HDAC5 was involved in the sensitivity to doxoru-

## Formononetin sensitizes glioma cells via HDAC5



**Figure 4.** Formononetin treatment decreased the expression of HDAC5. U87MG cells were treated with doxorubicin or in combination with formononetin (100  $\mu$ M) for 48 h. Western blot was performed to determine the expression of HDAC5 in glioma cells incubated with doxorubicin (A and B) or in combination with formononetin (100  $\mu$ M) (C and D). U87MG cells were transfected with the plasmid encoding HDAC5 (E) and cell viability was determined by CCK-8 methods after incubation with doxorubicin alone or combined with formononetin (F). \* $P < 0.05$ ; \*\* $P < 0.01$ .

bicin in glioma. Western blotting showed the upregulation of E-cadherin and downregulation of vimentin in HDAC5 siRNA-transfected U87MG cells (Figure 5D and 5E). Taken together, these data demonstrated that knockdown of HDAC5 by siRNA could alter the doxorubicin-induced EMT in glioma cells.

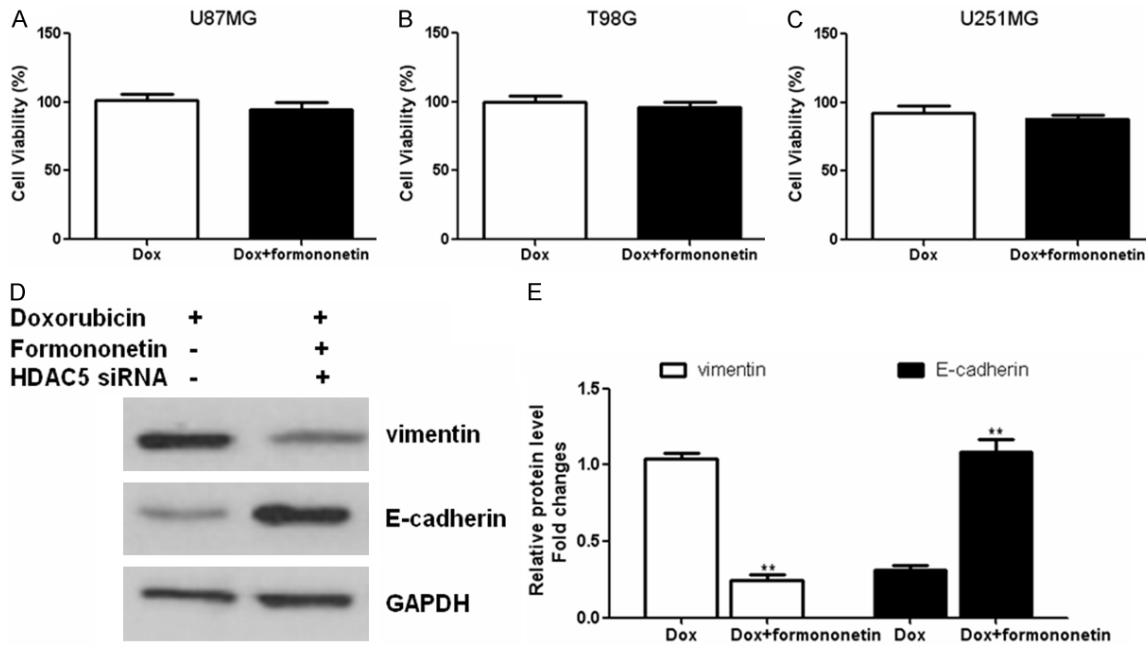
### Discussion

Accumulating evidences suggest that the acquired drug resistance to traditional chemotherapeutics has become a major obstacle to the triumph of chemotherapy [18]. In this study, we examined whether formononetin could

enhance the cytotoxicity of doxorubicin in human glioma cells. Our data showed that combination treatment with formononetin increased the doxorubicin sensitivity in glioma cells.

Many studies have indicated that EMT plays a key role in carcinogenicity, metastasis, progression and acquired chemoresistance in various types of cancers [19, 20]. During EMT, epithelial markers such as E-cadherin decrease, while mesenchymal markers such as vimentin increase [21]. In our study, we found that doxorubicin treatment induced EMT in glioma cells. However, formononetin co-administration reversed the EMT induced by doxorubicin in glioma

## Formononetin sensitizes glioma cells via HDAC5



**Figure 5.** Knockdown of HDAC5 diminished the doxorubicin-induced EMT in glioma cells. The HDAC5 siRNA-transfected glioma cells were incubated with doxorubicin alone or in combination with formononetin for 48 h. CCK-8 assay was used to determine the cell viability in different glioma cell lines including U87MG (A), U251MG (B) and T98G (C). Western blot analysis of E-cadherin and vimentin expression in HDAC5-siRNA or negative-siRNA transfected glioma cells (D and E). Relative protein expression of HDAC5 was quantified by band density with GAPDH served as control. \* $P < 0.05$ ; \*\* $P < 0.01$ .

ma cells. These results demonstrated that formononetin regulated doxorubicin-induced EMT in glioma cells.

The histone deacetylase (HDACs) family contains a family of 18 proteins and these proteins are classified into classes I-IV based on their homology and structure [22]. Accumulating evidences suggested that HDAC family functions as an important regulator in tumor progression and metastasis [23]. Recently, several HDAC inhibitors have been shown to exhibit anti-tumor activity in cancer cells and animal models [24]. In addition, clinical studies have also shown the potential application of HDAC inhibitors as anti-cancer agents [25]. HDAC5, a member of the class II histone deacetylase family, has been shown play critical roles in cell proliferation, cell cycle progression and apoptosis [26-28]. A recent study reported that HDAC5 promoted the twist 1 expression and highlighted a potential link between HDAC5 and osteosarcoma progression [29]. Our previous study demonstrated that HDAC5 promoted glioma cells proliferation via up-regulation of Notch 1 expression and might provide novel therapeutic targets in the treatment of gliomas [17]. In the

current research, we found that the expression of HDAC5 was significantly up-regulated in doxorubicin-treated glioma cells, which was reduced after formononetin co-treatment. In addition, overexpression of HDAC5 diminished the inhibitory effects of formononetin on glioma cells, suggesting that formononetin sensitized tumor cells through inhibition of HDAC5.

Furthermore, we investigated the molecular mechanism underlying the reversal of EMT by formononetin. The etiology of glioma involves a complex interplay of various factors, of which the accumulation of oncogenes and loss of tumor repressors are crucial events in the initiation and progression of cancer [30, 31]. Recently studies found that HDAC5 was aberrantly expressed in several types of tumor cells, such as glioma [17], osteosarcoma [29] and colon cancer [32]. In our previous study, we measured HDAC5 expression in glioma tumor tissues and showed that HDAC5 plays an important role in cell proliferation [17]. In the current study, our data showed that knockdown of HDAC5 by siRNA could alter cell phenotypes, suggesting that HDAC5 was a key factor in doxorubicin-induced EMT in glioma cells.

In conclusion, the present study showed that combined treatment with formononetin enhances the cytotoxicity of doxorubicin in glioma cells through suppressing HDAC5 and preventing doxorubicin-induced EMT. Therefore, combination therapy with formononetin may contribute to a better therapeutic effect in doxorubicin-based chemotherapy for patients with gliomas.

**Disclosure of conflict of interest**

None.

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## Formononetin sensitizes glioma cells via HDAC5

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