LABORATORY INVESTIGATION

Plumbagin induces growth inhibition of human glioma cells by downregulating the expression and activity of FOXM1

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Abstract Plumbagin, a natural quinonoid constituent isolated from the root of medicinal plant *Plumbago zeylanica* L, has exhibited anti-tumor and anti-proliferative activities in various tumor cell lines as well as in animal tumor models. However, its anticancer effects and the mechanisms underlying its suppression of glioma cell growth have not been elucidated. Oncogenic transcription factor Forkhead Box M1 (FOXM1) has garnered particular interest in recent years as a potential target for the prevention and/or therapeutic intervention in glioma, nevertheless, less information is currently available regarding FOXM1 inhibitor. Here, we reported that plumbagin could effectively inhibit cell proliferation, migration and invasion and induce apoptosis of glioma cells. Cell cycle assay showed that plumbagin induced G2/M arrest. Interestingly, we found that plumbagin decreased the expression of FOXM1 both at mRNA level and protein level. Plumbagin also inhibited the transactivation ability of FOXM1, resulting in down-regulating the expression of FOXM1 downstream target genes, such as cyclin D1, Cdc25B, survivin, and increasing the expression of $p21^{CIP1}$ and $p27^{KIP1}$. Most importantly, down-regulation of FOXM1 by siFOXM1 transfection enhanced plumbagin-induced change in viability. On the contrary, over-expression of FOXM1 by cDNA transfection reduced plumbagin-induced glioma cell growth inhibition. These results suggest that plumbagin exhibits its anticancer activity partially by inactivation of FOXM1 signaling pathway in glioma cells. Our findings indicate that plumbagin may be considered as a potential natural FOXM1 inhibitor, which could contribute to the development of new anticancer agent for therapy of gliomas.

Keywords Gliomas · Plumbagin · FOXM1 · Cell cycle · Apoptosis

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Introduction

Glioblastoma multiforme (GBM) is the most common form of malignant brain cancer in dults. The conventional therapy for GBM over the past decades has been surgery, radiotherapy and chemotherapy. However, the treatment outcome is still not satisfied owing to the rapid progression, high invasiveness and resistance to radiotherapy and chemotherapy [1, 2]. Therefore, understanding the mechanisms of gliomas development is critical for designing novel effective and safe drugs for treating the malignant gliomas.

Forkhead box M1 (FOXM1), the proliferation-specific oncogenic transcription factor, is a critical regulator of cell cycle progression, and has been reported to regulate the transcription of cell cycle-related genes essential for G_1/S and G_2/M transition [3, 4]. This evidence suggests that

activation of FOXM1 signaling is closely associated with carcinogenesis. FOXM1 signaling is frequently up-regulated in GBM, and can serve as an independent predictor of poor survival [5]. Recently, Zhang et al. have reported that FOXM1 plays a critical role in GBM-initiating cells (GICs) self-renewal and differentiation [6]. Another study has shown that recurrent GBM cell lines expressed higher levels of FOXM1 were resistant to TMZ. FOXM1 knockdown sensitized recurrent GBM cells to TMZ cytotoxicity [7]. These observations suggested that FOXM1 is an attractive target for the prevention and/or therapeutic intervention in GBM. However, less information is currently available regarding the role of FOXM1 inhibitor in GBMs.

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), a natural small molecular quinonoid constituent isolated from the root of the plant *Plumabago zeylanica*, has been exhibited several pharmacological and biological activities, such as anti-atherosclerotic, anti-bacterial, anti-inflammation and anticancer effects [8–10]. It has been reported that plumbagin can exert significant anti-proliferative and pro-apoptotic properties in various tumor cell lines cultured in vitro and animal tumor models in vivo [10–13]. Plumbagin can also inhibit invasion and migration of several cancer cell lines [14, 15]. Previous studies have suggested that the action mechanisms of plumbagin include inhibition of NF-κB and Akt/mTOR pathway, cell cycle arrest, disruption of microtubule network and generation of reactive oxygen species [11, 13, 16]. However whether plumbagin has effectiveness on the treatment of gliomas and its underlying action mechanisms still have not been investigated.

Herein, for the first time, we reported that plumbagin could inhibit expression and transcriptional activity of FOXM1, resulting in the growth inhibition of glioma cells, and induce cell apoptosis and cell cycle arrest. These results suggested that plumbagin may be considered as a potential anticancer agent for therapy of gliomas by inhibiting of FOXM1 activity.

Materials and methods

Cell culture and reagents

Human glioma cell lines SHG-44, A172 and U251were cultured in DMEM/F12 supplemented with 10 % FBS. These cell lines were incubated at 37 °C in a 5 % CO_2 humidified incubator. Primary antibodies against FOXM1, p21^{CIP1}, p27^{KIP1} and Cdc25B were obtained from santa cruz Biotechnology. The antibodies to survivin, cyclin D1 and β-actin were purchased from cell Signaling Technology. Plumbagin and all other chemicals were purchased from Sigma-Aldrich.

Plasmids

The vector (pDONR223-FOXM1), containing the full-length FOXM1 cDNA was obtained commercially from Yingrun Biotechnologies Inc. (Changsha,China). FOXM1 expression vector (p3 × FLAG-FOXM1) used in this study was constructed as follows: the FOXM1 cDNA was amplified using specific primers (sense: 5′-ATAAGAATGCGGCCGCATG AAAACTAGCCCCCGTCG-3′; antisense: 5′-CGCGGAT CCCTGTAGCTCAGGAATAAACTGG-3′) and subcloned in the p3 × FLAG-CMV-14 vector at the *NotI* and *BamH*I restriction sites. The FOXM1 binding site reporter plasmid (6 × FOXM1-luc) containing TACGTTGTTATTTGTT TTTTTCG repeated six times was constructed according to previous description [17].

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The cytotoxicity of plumbagin to SHG-44, A172 and U251 cells treated with concentrations of 0.625, 1.25, 2.5, 5, 10 and 20 μmol/L for 24, 48 and 72 h was assessed by the MTT assay as described elsewhere [14].

Cell proliferation and clonogenic assay

Cell proliferation was assessed by 5-ethynyl-2′-deoxyuridine (EdU) fluorescence staining using the Cell-Light^{TM} EdU DNA cell proliferation kit (Ruibo Biotech, Guangzhou, China). To determine long-term effects, SHG-44 cells were treated plumbagin for 3 h. Then, clonogenic assay was used to elucidate the possible differences in long-term effects of plumbagin on glioma cells as described elsewhere [13].

Cell cycle and apoptosis assays

To determine cell cycle distribution after plumbagin treatment, cell cycle analysis was carried out in SHG-44 cells treated with plumbagin for 24 h using propidium iodide (PI) staining and flow cytometry as described elsewhere [13]. SHG-44 cells were treated with plumbagin for 24 h, and then were harvested and stained with PI and Annexin V. The cell apoptosis was examined by flow cytometry analysis.

Wound healing and invasion assay

To investigate the effects of plumbagin on glioma cell migration and invasion, SHG-44 cells were treated with various concentrations of plumbagin. Then, the in vitro wound healing and invasion assay was performed according to our previous reports [18, 19].

Western blot analysis

SHG-44 cells were treated with different concentrations of plumbagin. After 24 h of incubation, total protein extracts from treated or untreated cells were subjected to Western blot analysis as described elsewhere [20]. The expression patterns of FOXM1, Cdc25B, cyclin D1, survivin, p21^{CIP} and p27^{KIP} were detected using specific antibodies all and β-actin was used as the loading control.

Real-time reverse transcription-PCR (RT-PCR) analysis

The total RNA from untreated cells or plumbagin-treated cells was isolated using the Trizol reagent (Invitrogen). Reverse transcription was performed by Transcriptor First Strand cDNA Synthesis Kit (Roche). PCR reaction and primers used in this study were exactly the same as reported elsewhere [21].

Luciferase reporter assay

SHG-44 cells (at 80–90 % confluency) were co-transfected with 6 × FOXM1-luc and renilla luciferase reporter vector using Lipofectamine^{TM} 2000 (Invitrogen). After incubation for 24 h, transfected cells were treated with 0.1 % DMSO or different concentrations of plumbagin, and then incubated for 24 h. Luciferase reporter assay was performed using the Dual Luciferase Assay kit (Promega). The activity of renilla luciferase was used as a transfection and expression efficiency control for the experiments. All samples were assayed in triplicate.

Small interfering RNAs (siRNAs) and plasmid transfection

SHG-44 cells were transfected with FOXM1 siRNA, siRNA negative control (siNC) and cDNA plasmid (p3 × FLAG-FOXM1), respectively, using Lipofectamine^{TM} 2000. The sequence of siRNAs (Shanghai GenePharma Co.) was listed below: siFOXM1: 5′-GGACCACUUUCCC UACUUU-3′; siNC: 5′-UUCUCCGAACGUGUCACGU-3′. All the transfections were performed three times independently.

Statistical analysis

The experimental results were expressed as the mean values ± S.E.M. from three independent experiments, each performed in triplicate or more. Comparisons between untreated group and plumbagin-treated group were evaluated by Student's t test. A $P < 0.05$ was considered as statistically significant.

Results

Plumbagin decreases viability and inhibits proliferation of glioma cells

To assess the potential growth inhibition effect of plumbagin on glioma cells, three human glioma cell lines were cultured in the presence of plumbagin and cell viable was estimated using MTT assay. As shown in Fig. 1a, glioma cells pre-treated with 0.625, 1.25, 2.5, 5, 10 and 20 μM of plumbagin for 48 h induced a dose-dependent growth inhibition in U251, SHG-44 and A172 cell lines. Higher activity was observed toward SHG-44 cells, and the IC$_{50}$ values obtained were 3.22 ± 0.51 μM. These data suggest that plumbagin can efficiently inhibit viability of glioma cells.

We next tested the rates of cell proliferation by EdU fluorescence staining and clonogenic assay in plumbagin-treated cells. As shown in Fig. 1b, d, the proliferation of plumbagin-treated cells was reduced to 63.31 ± 5.57 and 41.3 ± 3.28 % at 4 and 8 μM, respectively ($P < 0.01$) compared with control group. Colony formation assay further demonstrated that the treatment of plumbagin suppressed cell proliferation of SHG-44 cells (Fig. 1c, e). Compared with control group, the numbers of colony formation were markedly decreased by 36.26 ± 1.29, 78.15 ± 2.09 and 89.41 ± 8.54 % at 2, 4 and 6 μM, respectively. Taken together, these results indicate that plumbagin could inhibit the proliferation of SHG-44 cells.

Plumbagin induces cell cycle arrest

To investigate the precise mechanism that lead to inhibit plumbagin-mediated cell growth, we evaluated cell cycle distribution after plumbagin treatment using flow cytometry. As shown in Fig. 2a, plumbagin-treated cells caused a marked accumulation of cells in the G2/M phase ($P < 0.05$) and a significant decrease in G0/G1 phase populations ($P < 0.05$) at a concentration of 4 μM for 24 h compared with control group. These results suggested that plumbagin induces G2/M arrest in SHG-44 cells, consistent with previous studies [13, 22].

Plumbagin induces glioma cell apoptosis

Plumbagin-induced apoptosis has been reported in a variety of types of cancer cells [11, 23, 24], but this has not been characterized in glioma cells. Thus, we also assessed the ability of plumbagin to induce apoptosis in SHG-44 cells by Annexin V/PI staining. As Fig. 2b showed, in SHG-44 cells, following incubation for 24 h, approximately 36.9 % cells underwent apoptosis when exposed to 6 μM plumbagin. These results clearly showed that plumbagin induces glioma cells apoptosis.

Fig. 1 Plumbagin decreases viability and inhibits proliferation of glioma cells. **a** Cell viability analysis of plumbagin treatment for 48 h by MTT. **b** Measurement of anti-proliferation effects of plumbagin by EdU incorporation assay. **c** Plumbagin inhibits the colony formation of SHG-44 cells. **d** and **e** Quantitative results of EdU incorporation assay and clonogenic assay. The numbers of proliferative cells or colony formation were normalized to that of the control group. All the *Data* are presented as mean ± SEM in three repeats (*P < 0.05, **P < 0.01)
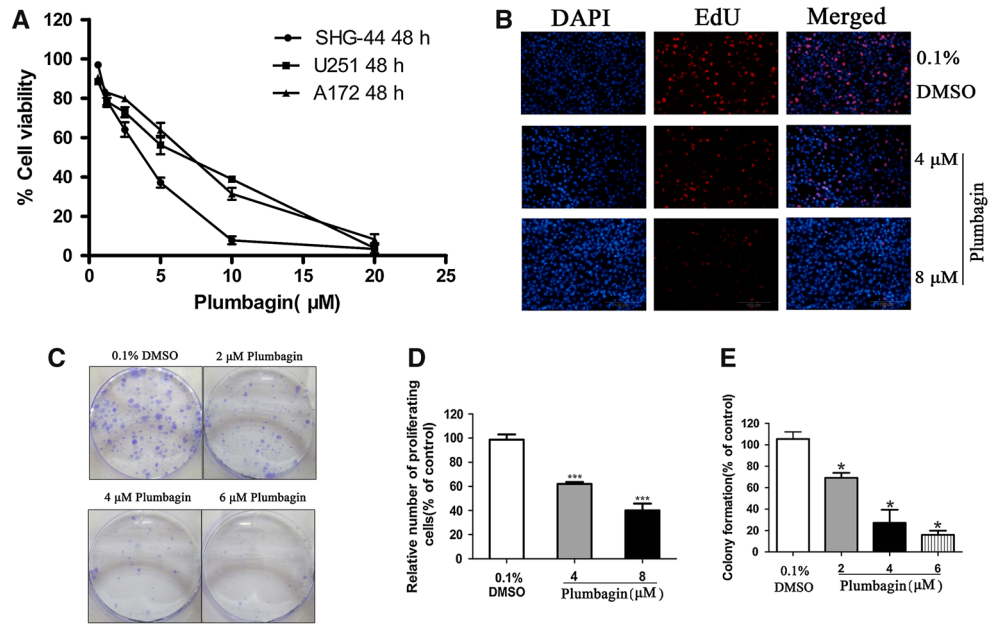

Fig. 2 Plumbagin induces cell cycle arrest and apoptosis of glioma cells. **a** Cell cycle distribution was assessed after plumbagin treatment by flow cytometry. Triplicate experiments were performed ($P < 0.05$). **b** Representative flow cytometry analysis of cell apoptosis co-stained with Annexin V/PI after plumbagin treatment for 24 h
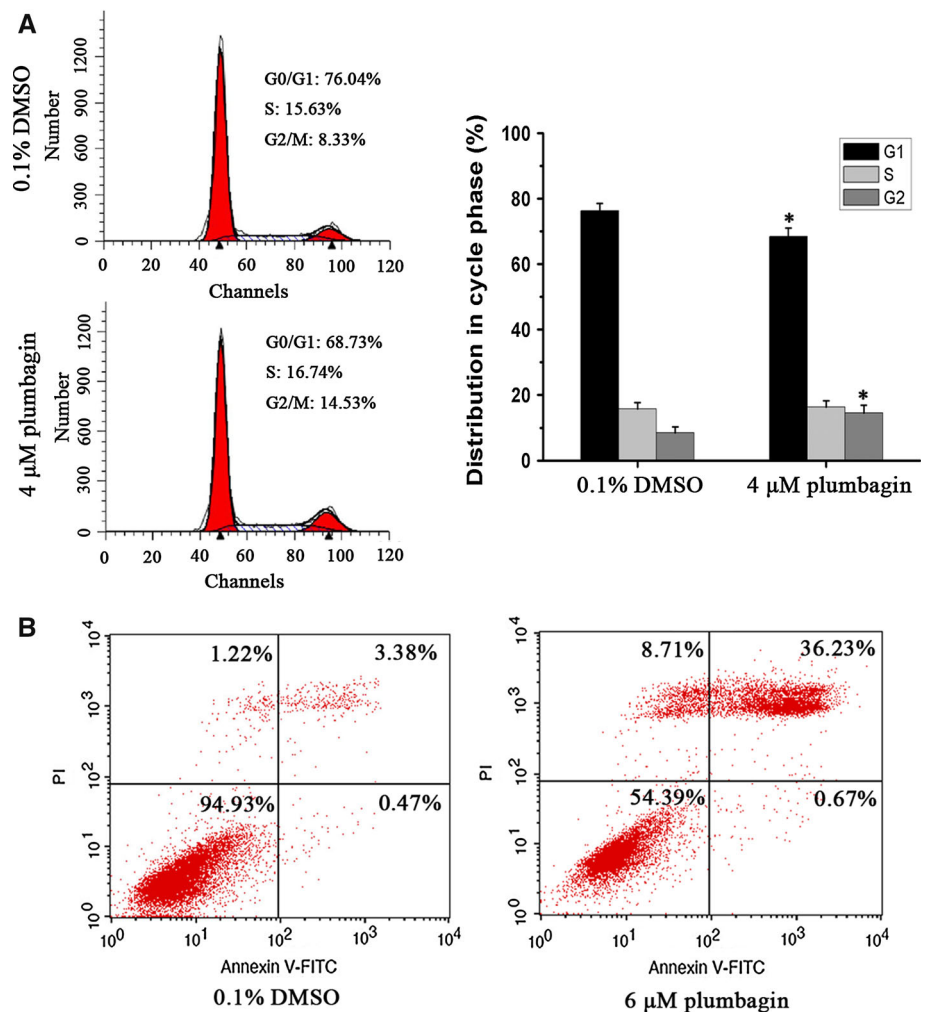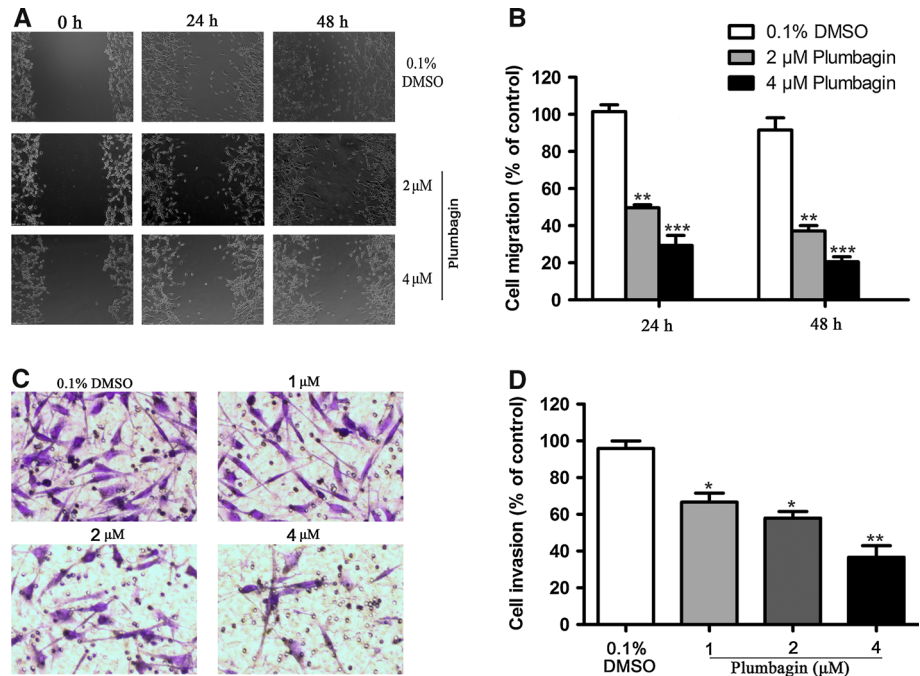
Fig. 3 Plumbagin inhibits the migration and invasion of SHG-44 cells. **a** Effect of plumbagin on SHG-44 cell migration as examined by wound healing assay. **c** Effect of plumbagin on SHG-44 cell invasion ability as examined by a transwell migration assay. **b** and **d** Quantitative analysis of migratory and invading cell numbers. The numbers of migratory or invading cells were normalized to that of the control group. The results are expressed as the mean ± SEM from three independent experiments (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

Plumbagin inhibits the migration and invasion of SHG-44 cells

In addition, we also detected whether plumbagin had inhibitory effects on migration and invasion of SHG-44 cells using in vitro wound healing and invasion assays. As is shown in Fig. 3a, b, we observed that 24 h after being scratched, compared with control group, the migratory cell numbers of plumbagin treatment group were decreased by 50.65 ± 3.96 and 69.60 ± 5.91 % at 2 and 4 μM, respectively. Furthermore, 48 h after being scratched, the migratory cell numbers of plumbagin treatment group had already decreased by 60.34 ± 6.58 and 78.32 ± 5.06 % at 2 and 4 μM, respectively. Consistent with this, the invasion assay showed that plumbagin induced a dose-dependent reduction of invasive cell numbers with increasing concentration of plumbagin. Compared with control group, the invasive cell numbers were reduced by 59.14 ± 6.78 % at 4 μM (Fig. 3c, d). Above these data indicate that plumbagin inhibits the migration and invasion of SHG-44 cells.

Down-regulation of FOXM1 inhibits glioma cell growth

Previous studies have suggested that FOXM1 signaling is over-expressed in gliomas and is closely associated with promotion of glioma cells growth and invasion [5, 25]. In our study, we characterized the expression level of FOXM1

in five glioma cell lines. As seen in Fig. 4a, compared to other glioma cell lines, the FOXM1 expression is highly expressed in SHG-44 cells. Next, we used FOXM1 specific siRNA (siFOXM1) to down-regulate the expression of FOXM1 (Fig. 4b) and to evaluate the effects of FOXM1 on the growth of SHG-44 cells by MTT assay. The results revealed that the cell numbers decreased by 38.9 % in SHG-44 cells after siFOXM1 transfection for 96 h compared to siNC group (Fig. 4c). Our data, together with previous reports, demonstrated the critical role of FOXM1 in glioma cell growth.

Effects of plumbagin on FOXM1 and its downstream target genes

To investegate whether FOXM1 is a downstream signaling target of plumbagin, we tested the FOXM1 mRNA and protein expression in SHG-44 cells treated with plumbagin using real-time RT-PCR and Western blotting analysis, respectively. As shown in Fig. 4d, e, we found that FOXM1 in plumbagin-treated SHG-44 cells were down-regulated both at mRNA and protein levels.

To confirm our results, we also examined the expression of FOXM1 downstream target genes. It is well known that FOXM1 regulates the transcription of cell cycle genes, such as cyclin D1, Cdc25B, survivin, p21[CIP1] and p27[KIP1] [26–28]. Western blot assay showed that plumbagin inhibited the expression of cyclin D1, Cdc25B, survivin and promoted the expression of p21[CIP1] and p27[KIP1]

Fig. 4 Effects of plumbagin on FOXM1 expression and activity. **a** The expression analysis of FOXM1 in five human glioma cell lines. **b** The downregulation efficacy of FOXM1 siRNA in SHG-44 cells. **c** The inhibition of cell growth after FOXM1 down-regulation. **d** The relative FOXM1 mRNA expression after plumbagin treatment as measured by real time RT-PCR. **e** FOXM1 protein level as examined by Western blot in plumbagin-treated SHG-44 cells. **f** Modulation of the protein expressions of FOXM1 downstream target genes by plumbagin. **g** Plumbagin inhibits the transactivation ability of FOXM1. Relative FOXM1 luciferase activity normalized with respect to corresponding renilla luciferase activity is shown. All the Data are presented as mean ± S.E.M. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the untreated group (0.1 % DMSO)
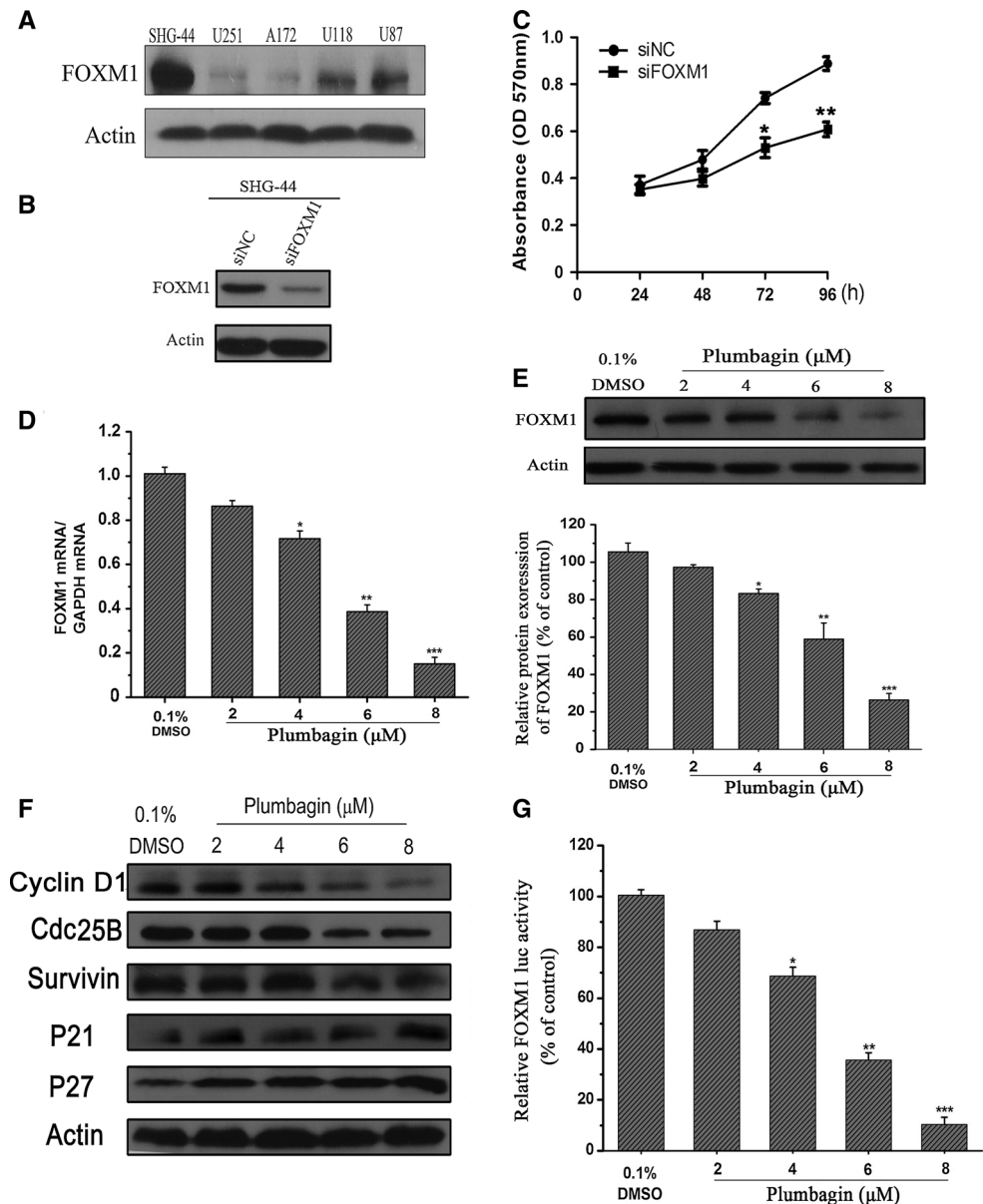
(Fig. 4f). Above these data indicated that FOXM1 may be as a downstream signaling target of plumbagin in gliomas.

**Plumbagin inhibits the transactivation ability of FOXM1**

To further elucidate the influence of plumbagin on FOXM1 activity and its downstream effectors, we used a FOXM1-luciferase construct containing the consensus binding site for FOXM1 to measure the effects of plumbagin on transactivation ability of FOXM1 using luciferase reporter assay. In a co-transfection study initiated in SHG-44 cells, we observed that plumbagin triggered a dose-dependent decrease on FOXM1-dependent reporter activity. At 8 µM, the relative FOXM1 luciferase activity reduced by about

10-fold compared with control group (Fig. 4g). Our data suggested that plumbagin regulated the expression of FOXM1 downstream effectors through inhibiting the transcription of target genes regulated by FOXM1.

Plumbagin inhibits glioma cell growth through down-regulation of FOXM1

In order to further confirm plumbagin inhibits the growth of glioma cells through downregulating FOXM1, SHG-44 cells were transfected with siFOXM1 or FOXM1 cDNA to measure whether it could reverse the cell viability we observed with plumbagin treatment by MTT assay. As seen in Fig. 5a, b, down-regulation of FOXM1 by siRNA transfection showed less expression of FOXM1 protein in

Fig. 5 Plumbagin inhibits glioma cell growth by down-regulating FOXM1. **a** and **c** The expression of FOXM1 was detected by Western blot to check the FOXM1 siRNA and cDNA plasmid transfection efficacy, respectively. **b** and **d** Cell viability was measured by MTT assay. *$P < 0.05$, **$P < 0.01$, compared with the untreated group (0.1 % DMSO)
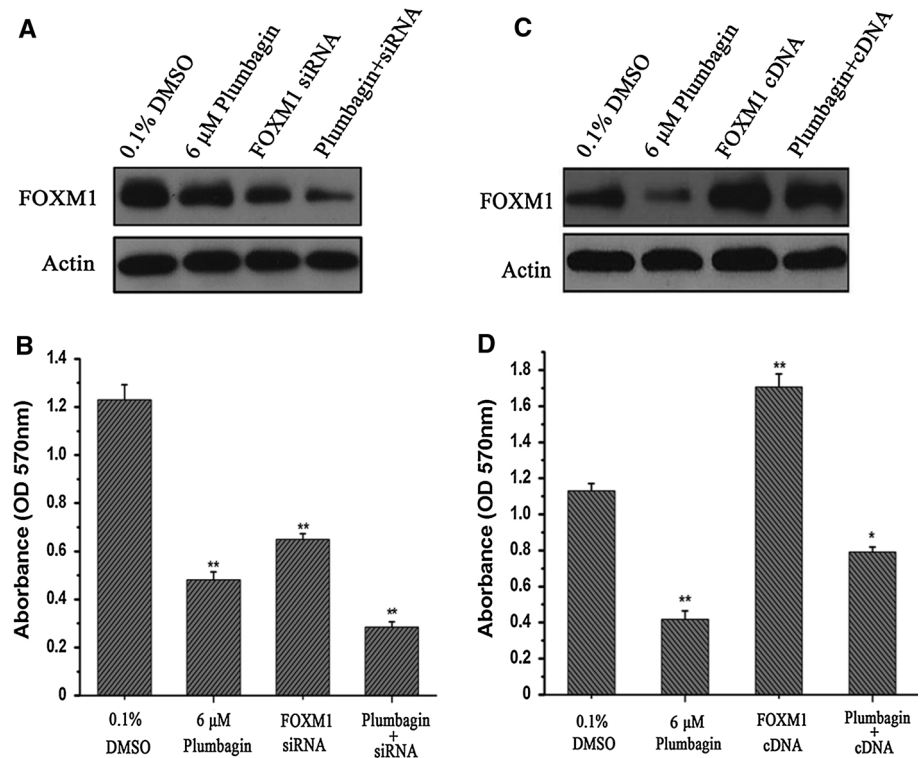
SHG-44 cells as confirmed by Western blot. MTT assay revealed that plumbagin plus FOXM1 siRNA inhibited cell growth to a greater degree compared with plumbagin alone (Fig. 5b). By contrast, up-regulation of FOXM1 by cDNA transfection displayed the overexpression of FOXM1 in SHG-44 cells (Fig. 5c), and showed that overexpression of FOXM1 could reduce plumbagin-induced change in viability (Fig. 5d). These findings provide a evidence suggesting that plumbagin-inhibited cell growth is partially attributed to inactivation of FOXM1 activity in glioma cells.

Discussion

Plumbagin, a potential anticancer agent, has been approved to significantly inhibit cell growth of various cancer cells [10–12]. In the present study, we found that plumbagin inhibits glioma cell growth and induces apoptosis of glioma cells. Consistent with previous studies [14, 15], we also found that plumbagin could inhibit glioma cell migration and invasion at lower doses. However, it should be noted that low concentration of plumbagin may also inhibit glioma cell proliferation and thus decrease the cell migration ability. FOXM1 as an oncogene is closely associated with cell proliferation and apoptosis. Increasing studies have shown the over-expression of FOXM1 gene in a majority of solid tumors [29] including malignant gliomas [5]. Consistent with this, we found that down-regulation of FOXM1 inhibited SHG-44 cell growth, suggesting that FOXM1 appears to be an attractive target for anticancer therapy. It has been reported that FOXM1 could be down-regulated by some drugs, such as EGFR inhibitor gefitinib, thiostrepton and siomycin A [30–32]. Surprisingly, we found that plumbagin could inhibit the expression of FOXM1 both at mRNA and protein levels. Importantly, down-regulation of FOXM1 together with plumbagin treatment inhibited cell growth to a greater degree compared to plumbagin treatment alone. By contrast, overexpression of FOXM1 attenuated plumbagin-induced cell growth inhibition in SHG-44 cells. Together with these findings, we strongly believe that pulumbagin-mediated cell growth inhibition is partially attributed to the inactivation of FOXM1 in glioma cells.

The importance of FOXM1 with respect to cell cycle is well recognized. FOXM1 promotes cell growth and tumorigenesis by activating regulating a series of cell cycle genes, including cyclin D1, Cdc25B and p21^{CIP1} [26, 27]. Wang et al. have reported that FOXM1 deficiency causes reduced expression of Cdc25B and elevated nuclear levels of the CDKI proteins p21^{Cip1} and p27^{Kip1} [3]. It is known that Cdc25B is the major effectors of the G2/M checkpoint response [33], and up-regulation of p21^{CIP1} has been reported to be associated with G2/M arrest in the cell cycle [34]. In our study, we also observed a marked reduction of Cdc25B in plumbagin-treated SHG-44 cells and the

increased expression of p21^{CIP1} (Fig. 4f). These results were strongly correlated with the altered cell cycle distribution phenotype and were similar to previous studies [13, 22]. Therefore, it may be indicated that FOXM1 affects the SHG-44 cell cycle by regulating the expression levels of Cdc25B, p21^{CIP1} and p27^{KIP1}. Bhat et al. have reported that proteasome inhibitors, such as MG115, MG132 and bortezomib could not only inhibit the expression of FOXM1, but they also suppress its transactivation ability [32]. Consistent with this, our results showed that plumbagin caused a dose-dependent decrease on FOXM1-dependent reporter activity, suggesting that plumbagin inhibits the expression of its downstream genes by suppressing transactivation ability of FOXM1. Our observations confirm earlier published results supporting the role of FOXM1 in glioma progression.

In summary, our results presented experimental evidence which strongly supports plumbagin may function as a potential natural inhibitor of FOXM1, ultimately causing cell growth inhibition, increment of cell apoptosis and cell cycle arrest. However, further in-depth studies are needed to confirm how plumbagin regulates the FOXM1 pathway, and some studies in vivo are also needed to further ascertain plumbagin-mediated anti-tumor activity by the inactivation of FOXM1 in the future. Based on the evidence provided here, we believe that plumbagin should be considered as a basis for the development of anticancer agents for glioma therapy.

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