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Natural Diterpenoid Isoferritin A (IsoA) Inhibits Glioma Cell Growth and Metastasis via Regulating of TGFβ-Induced EMT Signal Pathway

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: Malignant glioma is intractable primary brain carcinoma that has a poor survival rate. Natural diterpenoid isoferritin A (IsoA) presents antitumor effects by regulating signal pathways in tumor cells. In the present study we investigated the inhibitory effects of IsoA on glioma cells.

Material/Methods: The potential molecular mechanism of IsoA-mediated glioma cell growth and metastasis were investigated using Western blot, gene knockdown, immunofluorescence, and immunohistochemistry.

Results: Results showed that IsoA significantly inhibits growth and metastasis of glioma cells in multiple preclinical settings. *In vitro* assay showed that IsoA (4 mg/ml) treatment significantly induced apoptosis of glioma cells. Mechanism analysis demonstrated that IsoA (4 mg/ml) treatment decreased TGFβ and regulated EMT markers expression in glioma cells. Reduced expression of TGFβ in glioma cells was closely correlated with inhibitory effects of IsoA on growth and metastasis of glioma cells. TGFβ overexpression promoted glioma cell growth and invasion. Results also showed that IsoA treatment significantly decreased Fibronectin and Vimentin and increased E-cadherin, while TGFβ overexpression abolished the regulation mediated by IsoA in glioma cells. *In vivo* assay showed that IsoA treatment inhibited tumor growth in a glioma-bearing mouse model.

Conclusions: Results indicate that IsoA could be regarded as a potential anti-cancer agent by regulating TGFβ-induced EMT signal pathway.

MeSH Keywords: Drug Screening Assays, Antitumor • Oligodendroglioma • Transforming Growth Factor beta1

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Background

Glioblastoma is one of the most aggressive human tumors and has a poor prognosis despite maximal multimodal therapy [1,2]. Clinical investigations showed that patients with advanced glioblastoma frequently have symptom of seizures and/or stroke, which increases the difficulty and risk of clinical treatments due to disease characteristics, including the appearance of vascular proliferation, aggressive invasion, and necrosis around human normal brain tissues [3–5]. Statistical review and meta-analysis has revealed that glioblastoma accounts for approximately 75% in all malignant tumors in the brain [6]. A review of treatment modalities for glioblastoma revealed that patients with recurrence can be offered re-intervention, participation in clinical trials, anti-angiogenic agents, or local electric field therapy, but without an evident impact on survival [7]. Biomarkers of relevance to prognosis may contribute to identifying the efficacy of anti-cancer treatments [8]. Although molecular-targeted therapies, immunotherapy, and gene therapy are promising tools currently under research, potential anti-glioblastoma signal pathways need to be further investigated to develop more efficient anti-cancer strategies.

Natural diterpenoid is a class chemical compounds of 4 isoprene polymerization [9,10]. More and more natural diterpenoid compounds have been found by scientists and the function of diterpenoid compounds have been explored in many preclinical settings [11,12]. Recently, oncologists have found that natural diterpenoid compounds present anti-cancer effects on human cancer cells by targeting cellular signal transduction molecules [13,14]. Xu et al. have suggested that Pharicin A could be regarded as a novel natural ent-kaurene diterpenoid, which induces mitotic arrest and mitotic catastrophe of cancer cells, as well improving paclitaxel-resistant Jurkat and U2OS cells by interfering with BubR1 function [12]. Interestingly, Shi et al. have indicated that a novel natural diterpenoid, JDA-202, can significantly inhibit the growth of EC109 tumor xenografts, without significant body weight loss or multi-organ toxicities via promoting apoptosis of esophageal cancer through targeting Peroxiredoxin I [15].

In this study, we analyzed the inhibitory effects of natural diterpenoid isoferritin A (IsoA) on growth and metastasis of glioma cells both *in vitro* and *in vivo*. We further explored the potential molecular mechanism mediated by IsoA in growth and metastasis of glioma cells. We found that IsoA can be regarded as a potential anti-glioma agent through affecting the TGFβ-induced EMT signal pathway.

Material and Methods

Ethics statement

This study was conducted in strict accordance with recommendations in the Chinese Guide for the Care and Use of Laboratory Animals. Experimental protocols were approved by the Committee on the Ethics of Animal Experiments Defense Research, and animal sciences and animal health products were used in strict accordance with the guidelines of the National Institutes of Health.

Cells culture

Cells from a glioblastoma human cell line (U251 cells) were obtained from the American Type Culture Collection (ATCC). U251 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen, CA, USA). U251 cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C.

Transfection of small-interfering RNA (Si-RNA)

All siRNAs molecules were obtained from Invitrogen (Shanghai, China), including Si-RNA- TGFβ(Si-TGFβ) and Si-RNA-vector (Si-vector). U251 cells (1×10^6) were transfected with 100 pmol of Si-TGFβ targeting TGFβ with Si-vector as control (Applied Biosystems), using the Cell Line Nucleofector Kit L (Lonza) following the manufacturer's procedure.

Endogenous overexpression of TGFβ

U251 cells were seeded in 6-well plates until 90% confluence and the medium was then removed. U251 cells were transfected by pedue12.4-TGFβ (pTGFβ) using lipofectamine 2000 (Sigma-Aldrich). Stable TGFβ-overexpression U251 cells were selected using the dihydrofolate reductase/glutamine synthetase GS/DHFR (Invitrogen) screening system [16].

MTT assay

U251 cells were incubated with IsoA (2.0, 4.0, and 6.0 mg/ml) in 96-well plates at 37°C. After 24-h, 48-h, and 72-h incubation, respectively, 20 µl of MTT solution (5 mg/ml; Ameresco Inc., Framingham, MA, USA) mixed with PBS solution was added to each well and incubated at 37°C for 4 h. Then, the culture medium was replaced with 100 µl of DMSO (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added into the wells. The OD value was measured at 450 nm using a microplate spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

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Real-time quantitative PCR (RT-qPCR)

Total mRNA was isolated from U251 cells by using RNA Easy Mini Extract Kit (Sigma-Aldrich, Gaithersburg, USA). Expressions of TGFβ, Fibronectin, Vimentin, E-cadherin, Bcl-2, Bcl-w, Caspase-3, and Bad in U251 cells were calculated using the RT-qPCR kit with β-actin expression as an endogenous control. All procedures were performed according to the manufacturer's instructions. The forward and reverse primers were obtained from Invitrogen (Invitrogen, Shanghai, China) and β-actin levels were measured as an internal control. The expression level of mRNA was defined as mRNA/β-actin, measured by using the $2^{-\Delta\Delta}$Cq protocol. PCR was repeated 3 times in triplicate.

Apoptosis assay

U251 cells were incubated with IsoA (4.0 mg/ml) for 48 h to analyze apoptosis rates of tumor cells. Following incubation with IsoA, the U251 cells were trypsinized and collected. Subsequently, the cells were washed 3 times for 20 min each time in cold PBS and adjusted to 1×10^6 cells/ml per tube. The cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated Annexin V (BD Biosciences, Franklin Lakes, NJ, USA) for 1 h at 4°C according to the manufacturer's protocol and analyzed with a FACScan flow cytometer (BD).

Tumor cell migration and invasion

U251 cells were treated with IsoA (4.0 mg/ml) for 48 h and the control group received no treatment. Migration and invasion of U251 cells was conducted in a 6-well culture plate with chamber inserts (BD Biosciences). For migration assays, 1×10^6 /well concentration of the U251 cells were placed into the upper chamber with the migration-coated membrane. For invasion assays, cells (1×10^6 /well) were placed into the upper chamber with the invasion-coated membrane. After 48-h incubation, the cells that migrated or invaded cells were fixed and stained for 30 min in a 0.1% crystal violet solution in PBS. Migration and invasion of U251 cells were counted in 3 randomly selected views using a light microscope.

Western blotting

U251 cells (TGFβ overexpression or knockdown) were treated with IsoA or PBS for 48 h and then homogenized and lysed with radioimmunoprecipitation assay lysis buffer (Invitrogen, Thermo Fisher Scientific, Inc.). The lysates were sonicated for 5 s on ice and centrifuged at 6000×g for 10 min at 4°C. The protein expression levels were determined by incubation with rabbit anti-human primary antibodies: TGFβ (1: 500, ab310113, Abcam), Fibronectin (1: 500, ab2413, Abcam), Vimentin (1: 500, ab92547, Abcam), E-cadherin (1: 500, ab76055, Abcam), and β-actin (1: 500, ab8227, Abcam) for 12 h at 4°C following incubation with HRP-labeled IgG (1: 500, Abcam, Shanghai, China) for 2 h at 37°C. All proteins were measured using a chemiluminescence detection system.

Animal study

Specific pathogen-free male Balb/c mice (6–8 weeks old, 28–32 g body weight) were purchased from Slack Co., LTD (Shanghai, China). Nude mice were subcutaneously implanted with U215 cells (1×10^8) at subcutaneous sites and experimental mice were divided into 2 groups (n=30 in each group). The interventions were started on day 3 after tumor implantation (diameter: 5–6 mm). Tumor-bearing mice were intravenously injected with IsoA (4 mg/kg) and controls were injected with PBS. The treatment was continued 15 times every day. The tumor volumes were calculated to access the efficacy of Tunicamycin for tumor inhibition according to a previous study [17].

Immunohistochemistry

Tumors from xenograft mice were fixed by using formaldehyde (10%) and then were embed in paraffin. Immunohistochemical staining was performed using a Vidin-biotin-peroxidase technique on tumor tissues obtained from the mice. Paraffin-embedded tissue sections (4-μm-thick) were prepared and epitope retrieval was performed for further analysis. The paraffin sections were incubated with hydrogen peroxide for 10–15 min at 37°C and subsequently blocked with a regular blocking solution (5% skim milk powder) for 10–15 min at 37°C. Finally, the sections were incubated with biotinylated goat anti-human TGFβ (1: 500, ab310113, Abcam) Fibronectin (1: 500, ab2413, Abcam), Vimentin (1: 500, ab92547, Abcam), and E-cadherin (1: 500, ab76055, Abcam), respectively, at 4°C for 12 h. Samples were washed with PBS 3 times and relative expression of proteins was analyzed using a chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ).

Statistical analysis

All date are presented as the mean ± standard deviation of triplicate. Statistical evaluation was performed using the *t* test or one-way analysis of variance followed by Tukey HSD test using SPSS Statistics 19.0 and GraphPad Prism version 5.0 with the help of Microsoft Excel. * $P<0.05$ and ** $P<0.01$ were considered statistically significant differences.

Results

IsoA treatment inhibits growth and aggressiveness of glioma cells

We first analyzed inhibitory effects of IsoA on U251 growth and aggressiveness. As shown in Figure 1A, IsoA inhibited U251

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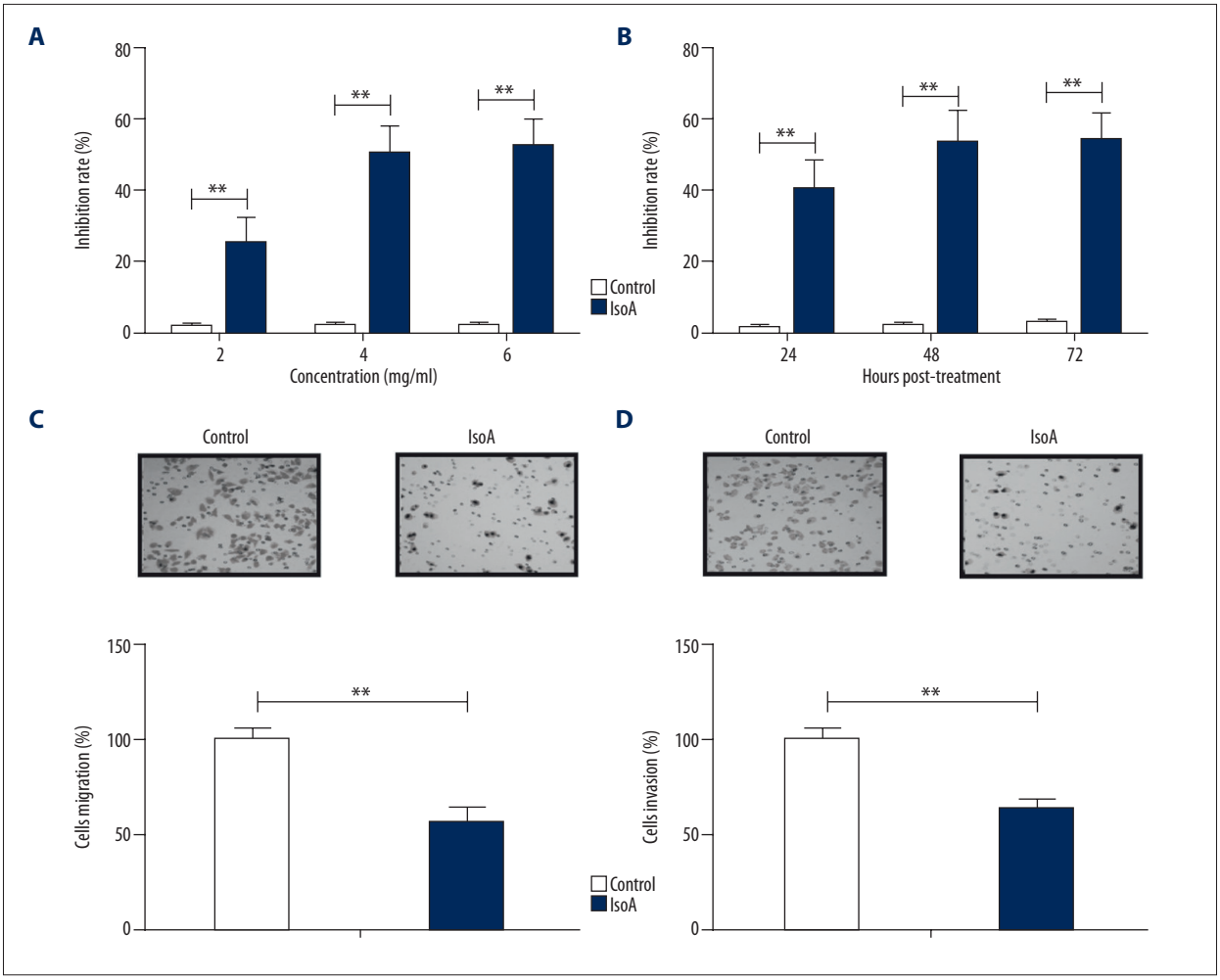

Figure 1. IsoA treatment significantly inhibits growth and aggressiveness of glioma cells. (**A**) IsoA inhibits U251 growth in a dose-depended manner (2, 4, and 6 mg/ml). (**B**) IsoA inhibits U251 growth a time-dependent (24, 48, and 72 h) manner. (**C, D**) IsoA (4 mg/ml) significantly inhibits migration (**C**) and invasion (**D**) of U251 cells after 48-h incubation.

growth in a dose-depended manner, and 4 mg/ml presented maximum toxicity for glioma cells. Results also demonstrated that IsoA inhibited U251 and A172 cell growth in a time-dependent (24, 48, and 72 h) manner (Figure 1B). We found that IsoA (4 mg/ml) significantly inhibited migration and invasion of U251 cells after 48-h incubation (Figure 1C, 1D). These results indicate that IsoA treatment can significantly inhibit growth and aggressiveness of glioma cells.

IsoA treatment promotes apoptosis of glioma cells through regulating apoptosis-related gene expression

Induction of apoptosis of tumor cells is the standard method used to evaluate the efficacy of anti-cancer drugs. We observed that IsoA treatment promoted apoptosis (Figure 2A) and decreased viability of U251 cells after 48-h incubation (Figure 2B). Results showed that IsoA treatment markedly inhibited anti-apoptosis gene Bcl-2 and Bcl-w gene expression in U251 cells (Figure 2C). We also found that pro-apoptosis gene caspase-3 and Bad gene expression levels were significantly increased in U251 cells (Figure 2D). These results suggest that IsoA treatment promotes apoptosis through decreasing expression of anti-apoptosis genes and increasing pro-apoptosis gene expression in glioma cells.

IsoA treatment decreases TGFβ and regulates EMT markers in glioma cells

TGFβ and EMT markers expression levels were analyzed in IsoA-treat glioma cells. As shown in Figure 3A, 3B, IsoA treatment (4 mg/ml) decreased TGFβ mRNA and protein expression levels in U251 cells. Results demonstrated that IsoA treatment decreased Fibronectin and Vimentin and increased E-cadherin mRNA and protein expression levels in U251 cells (Figure 3C, 3D). These results indicate that IsoA treatment can decrease TGFβ and regulate EMT markers in glioma cells.

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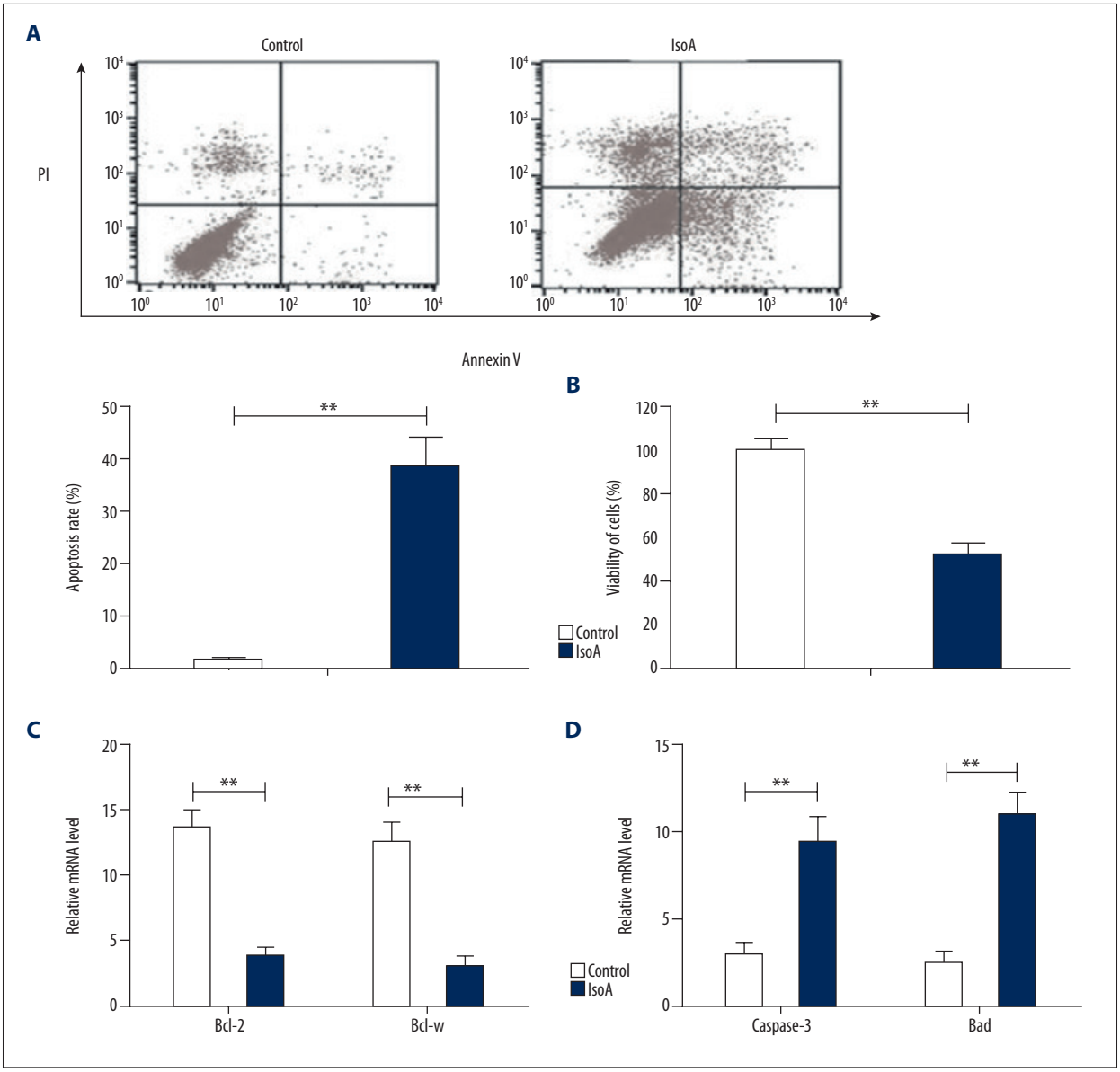

Figure 2. IsoA (4 mg/ml) treatment induces apoptosis of glioma cells. (**A**) IsoA treatment induces apoptosis of U251 cells after 48-h incubation. (**B**) IsoA treatment decreases viability of U251 cells after 48-h incubation. (**C**) IsoA treatment decreases anti-apoptosis gene Bcl-2 and Bcl-w gene expression in U251 cells. (**D**) IsoA treatment increases pro-apoptosis gene caspase-3 and Bad gene expression levels in U251 cells.

IsoA treatment regulates growth and aggressiveness of glioma cells through TGFβ-induced EMT signal pathway

To analyze the potential mechanism of IsoA-inhibited growth and aggressiveness of glioma cells, TGFβ was overexpressed or silenced in U251 cells. We observed that TGFβ knockdown (si-TGFβ) decreased Fibronectin and Vimentin and increased E-cadherin mRNA and protein expression levels in U251 cells (Figure 4A, 4B). TGFβ overexpression (pTGFβ) canceled IsoA-regulated (pTGFβ-IsoA) Fibronectin, Vimentin, and E-cadherin expression levels in U251 cells (Figure 4C, 4D). We found that TGFβ knockdown (Si-TGFβ) significantly inhibited growth and aggressiveness of U251 cells (Figure 4E–4G). Notably, TGFβ overexpression abolished IsoA-inhibited growth and aggressiveness of U251 cells (Figure 4H–4J). These results indicate that IsoA can regulate growth and aggressiveness of glioma cells through the TGFβ-induced EMT signal pathway.

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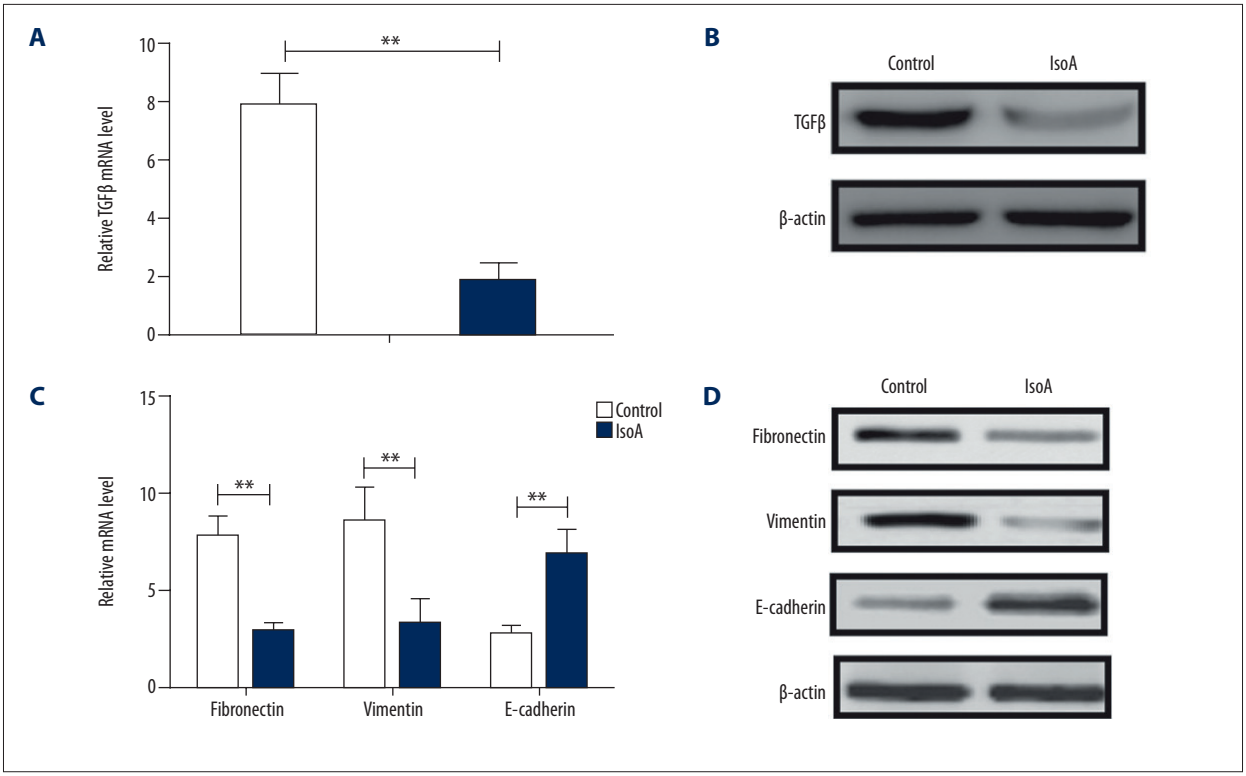
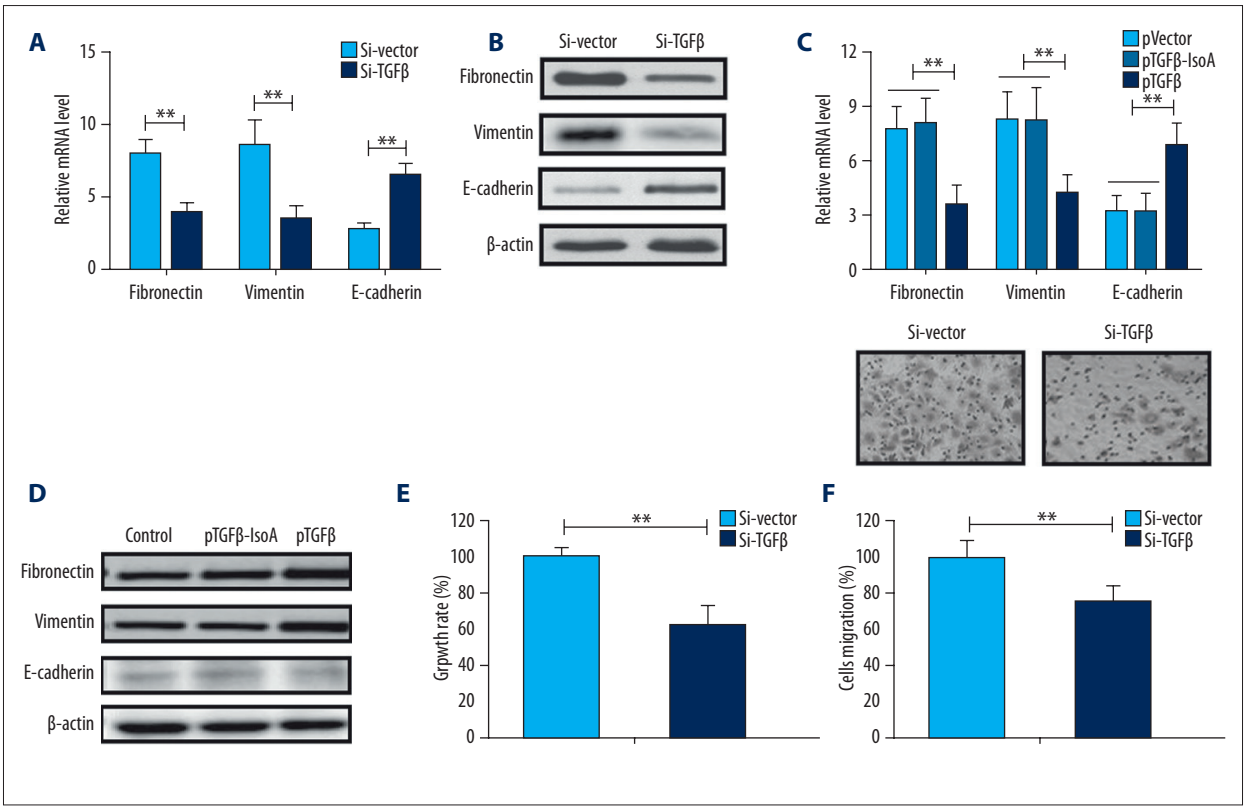


Figure 3. IsoA (4 mg/ml) treatment decreases TGFβ and regulates EMT markers in glioma cells. (**A, B**) IsoA treatment decreased TGFβ mRNA (**A**) and protein (**B**) expression levels in U251 cells. (**C, D**) IsoA treatment decreases Fibronectin, Vimentin, and increased E-cadherin mRNA (**C**) and protein (**D**) expression levels in U251 cells.



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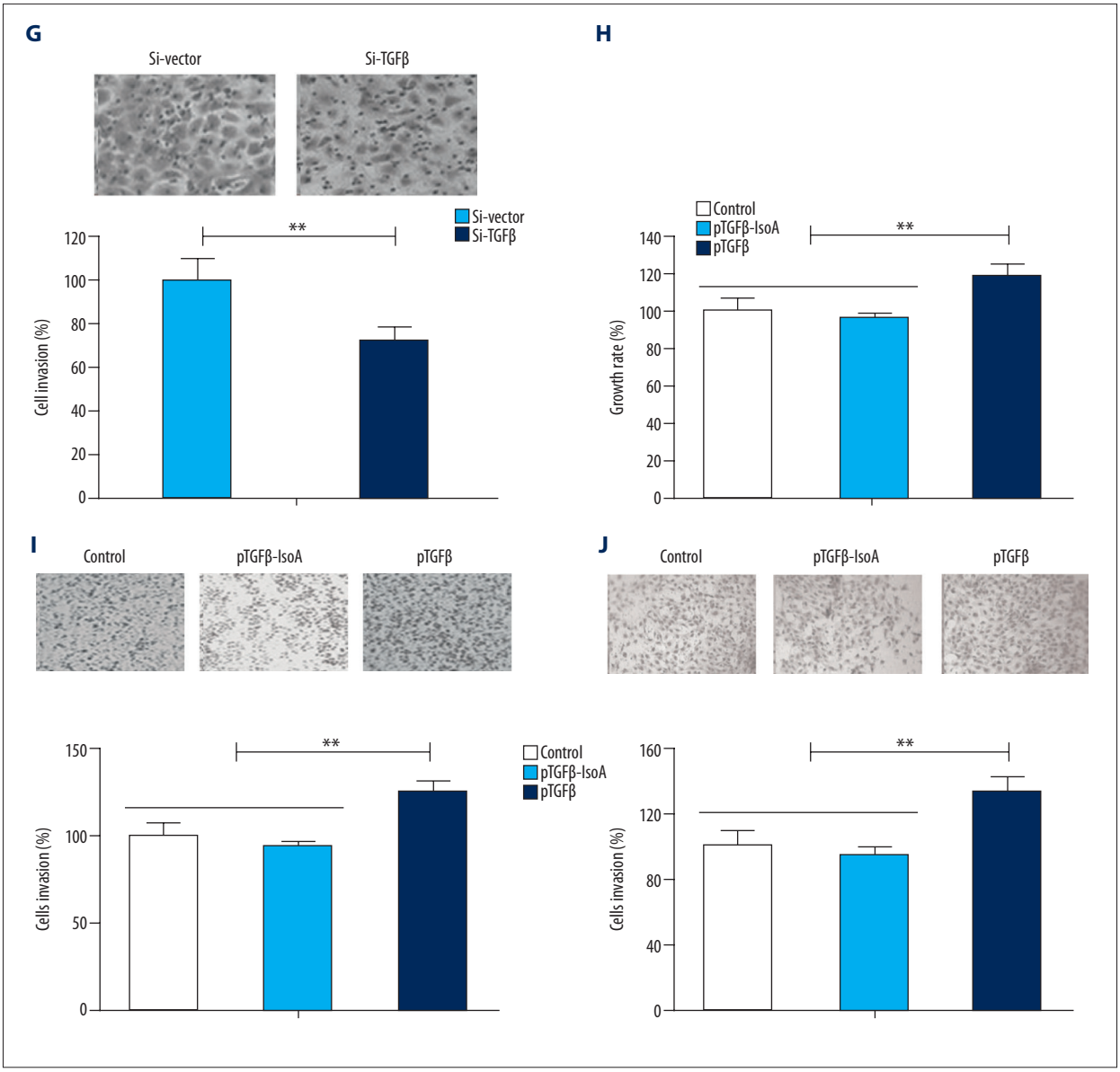

Figure 4. IsoA regulates growth and aggressiveness of glioma cells through TGFβ-induced EMT signal pathway. (**A, B**) TGFβ knockdown (Si-TGFβ) down-regulates Fibronectin and Vimentin and increased E-cadherin mRNA (**A**) and protein (**B**) expression levels in U251 cells. (**C, D**) TGFβ overexpression (pTGFβ) canceled IsoA-regulated (pTGFβ-IsoA) Fibronectin, Vimentin, and E-cadherin mRNA (**C**) and protein (**D**) expression levels in U251 cells. (**E–G**) TGFβ knockdown (Si-TGFβ) significantly inhibits growth (**E**), migration (**F**), and invasion (**G**) of U251 cells. (**H–J**) TGFβ overexpression abolishes IsoA-inhibited growth (**H**), migration (**I**), and invasion (**J**) of U251 cells

IsoA treatment significantly inhibits tumor growth in a glioma-bearing mouse model

We further investigated *in vivo* effects of IsoA on glioma tumor growth in a glioma-bearing mouse model. As shown in Figure 5A, IsoA treatment (4 mg/kg) significantly inhibited tumor growth in the mouse model. Immunohistochemistry demonstrated that IsoA treatment decreased TGFβ, Fibronectin, and Vimentin and increased E-cadherin expression levels in tumors (Figure 5B). These results showed that IsoA treatment can significantly inhibit tumor growth *in vivo*.

Discussion

Glioblastoma is a common and aggressive nervous system malignant tumor that seriously affects human health [18,19]. Current anti-cancer treatments have presented remarkable efficacy for

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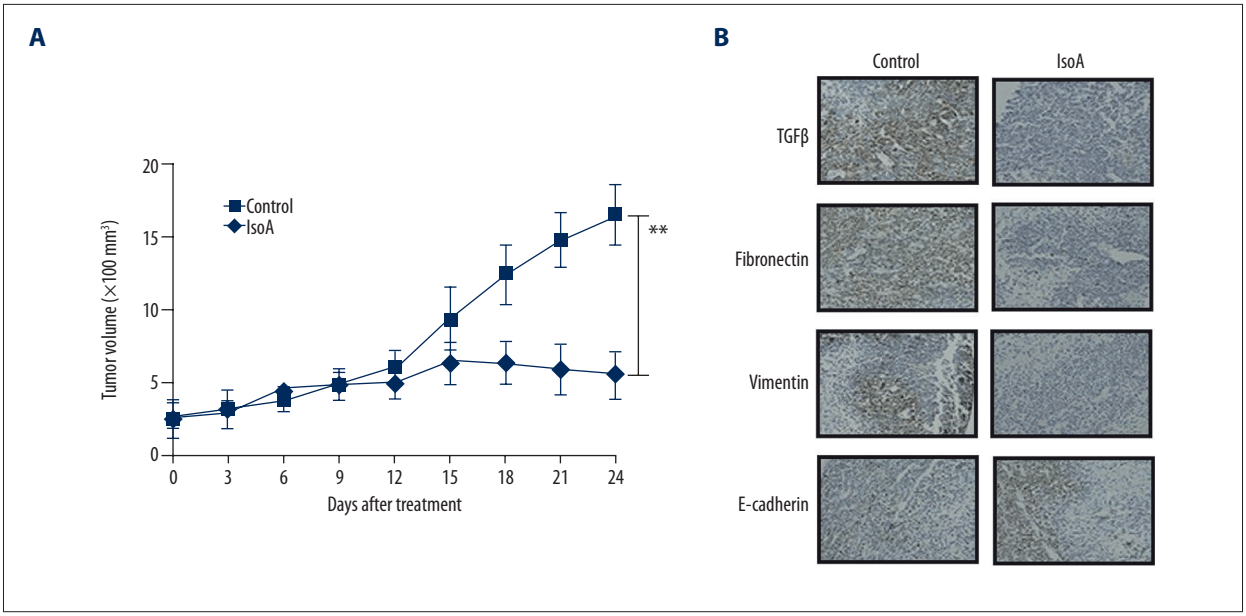

Figure 5. IsoA treatment significantly inhibits tumor growth in glioma-bearing mouse model. (**A**) IsoA treatment (4 mg/kg) significantly inhibited tumor growth in the U251-bearing mouse model. (**B**) IsoA treatment decreased TGFβ, Fibronectin, and Vimentin and increases E-cadherin expression levels in tumors as determined by immunohistochemistry.

glioblastoma therapy [20,21]. Interestingly, the anti-cancer effect of natural diterpenoid has been identified in different human cancer cells [12,22]. However, the potential signal pathways mediated by natural diterpenoid are not well understood yet. In this study, we analyzed the inhibitory effects of natural diterpenoid IsoA on glioblastoma both *in vitro* and *in vivo*. Our findings showed that natural diterpenoid IsoA not only significantly suppressed growth and metastasis of glioma tumor cells, but also promoted apoptosis of glioma cells. Notably, we found that IsoA can regulate growth and aggressiveness of glioma cells through the TGFβ-induced EMT signal pathway.

Many studies have investigated the anti-cancer efficacy of natural diterpenoid for various human cancer cells. One study showed that a diterpenoid derivative, NC043, is a novel small molecule that leads to inhibition of colorectal cancer cells growth by down-regulation of the canonical Wnt signaling pathway [23]. In this study, results indicated that IsoA treatment suppresses glioma cells growth and metastasis. Another study has suggested that cytotoxic esterified diterpenoid alkaloid derivatives could increase selectivity of a drug-resistant cancer cell line, which provides promising strategies for further development into anti-cancer agents [24]. In addition, diterpenoid can augment apoptosis of human laryngeal cancer cells by inhibition of caspase-9 [25]. We found that IsoA treatment induced apoptosis of glioma cells by regulating apoptosis-related gene expression. Furthermore, El Khaw et al. showed that the natural diterpenoid IsoA inhibits thioredoxin-1 and triggers potent ROS-mediated antitumor effects [26]. We further determined the potential signal pathway mediated by IsoA in glioma cells. Research shows that high TGFβ-Smad activity confers poor prognosis in glioma patients and promotes cell proliferation, depending on the methylation of the PDGF-B gene [27]. Gene set enrichment analysis has revealed that enrichment of the EMT pathway is a predictive biomarker for radiation-treated glioma patient prognosis. In the present study, we found that IsoA can regulate growth and aggressiveness of glioma cells through the TGFβ-induced EMT signal pathway. Additionally, regulation of EMT can lead to inhibition of cellular proliferation, migration, and invasion of the malignant glioma cell line [28]. We found that TGFβ overexpression regulated EMT marker expression, which led to abolishment of IsoA-regulated Fibronectin, Vimentin, and E-cadherin expression levels in U251 cells.

Conclusions

In conclusion, our study indicates that IsoA is as tumor-suppressor agent for glioma therapy. We found IsoA regulates growth, migration, and invasion of glioma cells through regulation of TGFβ-mediated EMT signaling pathways, which elucidates the molecular mechanism mediated by natural diterpenoid for cancer cells and may provide a potential strategy for the treatment of glioma.

Conflict of interest

None.

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