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Paclitaxel inhibits growth and proliferation of glioblastoma through *MMP-9*-meidated p38/JNK signaling pathway.

Dong Ruan[1], Xin Li[2], Aixia Li[3], Baohui Liu[4], Feng Xu[1*]

[1]Department of Neurosurgery, Xianning Central Hospital, the First Affiliated Hospital of Hubei University of Science and Technology, Xianning City, PR China

[2]Department of Computer Science and Technology College, Hubei science and Technology University, Xianning City, PR China

[3]Department of Encephalopathy, Xianning Hospital of Traditional Chinese Medicine, Xianning City, PR China

[4]Department of Neurosurgery, Renmin Hospital, Wuhan University, Wuhan City, PR China

Abstract

Glioblastoma is the most aggressive primary brain tumors originated from the glial cells in adults, which is characterized by stronger migration and invasion. Paclitaxel is an efficient anti-glioblastoma drug for patients in clinical. However, potential molecular mechanism of paclitaxel-inhibited growth and proliferation of glioblastoma has not been well understood yet. In this study, we investigated signal pathway mediated by paclitaxel in glioblastoma cells. Our results demonstrated that paclitaxel treatment inhibited growth and proliferation of glioblastoma cell line U251. Paclitaxel administration induced apoptosis of U251 cells by up-regulation of caspase signal pathway. Mechanism analyses showed that paclitaxel addition down-regulated *MMP-9* expression, which further inhibited p38/JNK signaling pathway in U251 cells. *In vivo* assays showed that Paclitaxel treatment could significantly inhibit glioblastoma growth and prolong survival of tumor-bearing mice. In conclusion, these results indicate Paclitaxel administration inhibits growth and proliferation of glioblastoma through *MMP-9*-meidated p38/JNK signaling pathway, which contributes to understanding signal pathway of paclitaxel-suppressed glioblastoma growth.

Keywords: Glioblastoma, Paclitaxel, Proliferation, *MMP-9*, p38/JNK.

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Introduction

Malignant glioma is the most common primary brain carcinoma and presents poor survival rate in the world, which is characterized by the appearance of vascular proliferation, aggressive invasion, and necrosis around human normal brain tissues [1-3]. Glioblastoma are the most common and lethal primary tumors of the central nervous system due to strong invasive and heterogeneous nature as well as resistance to multimodal treatments of anti-cancer drugs [4,5]. Glioblastoma exhibits a poor prognosis despite maximal multimodal therapy and the median survival of most patients is less than one year after application of multimodal therapies [6,7]. Therefore, understanding potential mechanism glioblastoma of growth and proliferation contributes to analyse signal pathway and explore more targets for the treatment of glioblastoma.

Paclitaxel is a tricyclic diterpene compound that loaded biodegradable poly-(DL-lactic-co-glycolic) acid (PLGA) foams with microporous matrix [8]. Paclitaxel presents better therapeutic effects for human cancer, such as ovarian cancer and breast cancer, lung cancer, colorectal cancer, melanoma, head and neck cancer, lymphoma, as well as brain tumors [9-11]. Woo et al. have paclitaxel can induce apoptotic cell death in human glioblastoma U87MG cells via regulation of apoptotic functions of p53 and c-Jun N-Terminal Kinase [JNK] [12]. In addition, human CD14+ cells loaded with paclitaxel inhibit glioblastoma cells proliferation *in vitro*, which suggest that Paclitaxel could be used to delivery anti-cancer immunotherapy drugs to kill glioblastoma cells [13]. Furthermore, Merighi et al. have suggested that paclitaxel-induced apoptosis through regulation of the extracellular signal-regulated kinase 1/2 activities, which is able to modulate *Bad* phosphorylation [11]. However, *MMP-9*-meidated p38/JNK signaling pathway in growth and proliferation mediated by Paclitaxel has not been investigated in the progression of glioblastoma.

In the current study, we analysed the potential mechanism of glioblastoma cells growth and proliferation during paclitaxel treatment. This study assumed that paclitaxel inhibited growth and metastasis of glioblastoma cells through regulation of

MMP-9-meidated p38/JNK signaling pathway. Furthermore, we provide strong evidences that Paclitaxel can be regarded as potential anti-cancer drug for the treatment of glioblastoma via inhibition of growth and proliferation.

Materials and Methods

Ethic statement

This analysis was performed by the recommendations in the Guide for the Care and Use of Laboratory Animals. All animals' protocols and animals were performed in accordance with National Institutes of Health and approved by Committee on the Ethics of Animal Experiments Defence Research.

Cells culture

U251 and U87MG cells were purchased from American Type Culture Collection (ATCC). All cells were cultured in DMEM (Sigma-Aldrich) medium (Gibco, CA, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen, CA, USA). All cells were cultured in a 37°C humidified atmosphere of 5 % CO_2.

MTT assay

U251 and U87MG cells were incubated with Paclitaxel (5, 10, 15 or 20 mg/ml) and or MMP inhibitor (ab142180, China) in 96 well plates for 24, 48 and 72 h in triplicate for each condition with PBS as control. After incubation, 20 µl of MTT (5 mg/ml) in PBS solution was added to each well, the plate was further incubated for 4 hours. Most of the medium was removed and 100 µl of DMSO was added into the wells to solubilize the crystals. The OD was measured by a BIO-RAD (ELISA) reader at wavelength of 450 nm.

MMP-9 overexpression

U251 and U87MG cells were cultured until 90% confluence and the media was then removed. U251 and U87MG cells were washed and transfected by lentivirus-*MMP-9* (p*MMP-9*) using lipfectamine 2000 (Sigma-Aldrich). Protein expression levels of purpose protein were analysed in *MMP-9*-overexpressed U251 and U87MG cells.

Real-Tome quantitative PCR (RT-qPCR)

Total RNA was extracted from U251 and U87MG and the identified RNA was applied to the cDNA synthesis by reverse transcription PCR. One-tenth of the cDNA was used to fluorescent quantitative RT-PCR by using iQ SYBR Green System. Relative multiples of change in mRNA expression was calculated by $2^{-\Delta\Delta Ct}$. The results are expressed as the n-fold difference relative to normal β-actin control.

Apoptosis assay

U251 and U87MG cells were incubated with paclitaxel (15 mg/ml) for 48 h. After incubation, all tumor cells were trypsinized and collected. Subsequently, U251 and U87MG cells were washed in cold PBS and adjusted to 1×10^6 cells/ml with PBS, labeled with annexin V-FITC and PI (Annexin V-FITC Kit, BD), and analysed with a FACScan flow cytometer (BD).

Western blotting

U251 and U87MG cells and tumors were homogenized in lysate buffer containing protease-inhibitor and were centrifuged at 8000 rpm/min at 4°C for 10 min. The purpose protein expression levels were incubated with rabbit anti-human primary antibodies anti-*MMP-9* (1:500; Cell Signaling, Danvers, MA, USA), anti-p38 (1:500; Cell Signaling, Danvers, MA, USA), anti-JNK (1:1000; Cell Signaling, Danvers, MA, USA), anti-phospho-JNK (Thr183/Tyr185) (1:1000; Cell Signaling), and anti-actin (1:5000; Millipore, Billerica, MA, USA). The following were used: HRP-labeled IgG (Abcam, Shanghai, China) for 1 h at 37°C. All proteins were visualized by using chemi-luminescence detection system.

Animal study

Specific pathogen-free male Balb/c mice were purchased from Slack co., LTD (Shanghai, China). Nude mice were subcutaneously implanted U251 (10^6) or U87MG (10^6) into experimental mice. Mice were divided into two groups (n=30). Treatments were initiated on day 6 after tumor implantation (diameter: 5-6 mm). Tumor-bearing mice were intravenously injected paclitaxel (10 mg/kg) with PBS as control. The treatment was continued 9 times once time a day. The tumor volumes were calculated to access the efficacy of Paclitaxel for tumor inhibition according to previous study [14].

Immunohistochemistry

Tumors from xenografted mice were fixed by using formaldehyde (10%) followed with embed in paraffin. Antigen retrieval was performed in tumor sections and the sections were incubated with rabbit anti-mouse primary antibodies anti-*MMP-9* (1:500; Cell Signaling, Danvers, MA, USA), anti-p38 (1:500; Cell Signaling, Danvers, MA, USA), anti-JNK (1:1000; Cell Signaling, Danvers, MA, USA). Tumor tissues were washed with PBS three times and incubated with biotinylated secondary antibodies anti-rabbit IgG (all 1:200; Pierce Biotechnology, Rockford, IL, USA). Biotin-peroxidase signals were detected using 0.5 mg/mL 3'3'-Diaminobenzidine (DAB)/0.003% H_2O_2 (Dako, Carpinteria, CA, USA) as a substrate. Results were recorded using a microscope (BX51; Olympus, Tokyo, Japan).

Statistical analysis

All date were expressed as mean ± SD of triplicate dependent experiments and analysed by using student t tests or one-way ANOVA (Tukey HSD test). All data were analysed 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 statistical differences.

Results

Paclitaxel inhibits growth and proliferation of glioblastoma cells in vitro

Paclitaxel exhibits anti-cancer efficacy for the treatment of glioblastoma. Results demonstrated that Paclitaxel inhibited glioblastoma cells growth in a dose-dependent manner (5, 10, 15 and 20 mg/ml; Figures 1A and 1B). Paclitaxel reached maximum inhibition for U251 and U87MG cells when concentration arrived at 15 mg/ml. We observed that paclitaxel (15 mg/ml) inhibited U251 and U87MG cells growth in a time-dependent manner (24, 47 and 72 h; Figures 1C and 1D). Results also demonstrated that paclitaxel administration significantly inhibited proliferation of U251 and U87MG cells (Figures 1E and 1F). These results suggest that paclitaxel can inhibit growth and proliferation of glioblastoma cells *in vitro*.

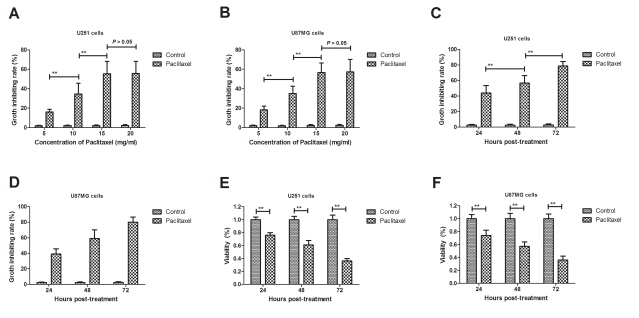

Figure 1. *Paclitaxel inhibits growth and proliferation of glioblastoma cells in vitro. (A, B) Paclitaxel inhibits U251 (A) and U87MG (B) cells growth in a dose-dependent manner (5, 10, 15 and 20 mg/ml). (C, D) Paclitaxel (15 mg/ml) inhibits U251 (C) and U87MG (D) cells growth in a time-dependent manner (24, 47 and 72 h). (E, F) Paclitaxel (15 mg/ml) inhibits U251 (E) and U87MG (F) cells growth in a time-dependent manner (24, 47 and 72 h).*

Paclitaxel induced apoptosis of glioblastoma cells in vitro

We analysed the efficacy of Paclitaxel on inducing apoptosis of glioblastoma cells. As shown in Figures 2A and 2B, Paclitaxel (15 mg/ml) significantly induced apoptosis of U251 and U87MG cells. Western blot demonstrated that showed that Paclitaxel treatment down-regulated anti-apoptosis gene *Bcl-2* and *Bcl-w* expression and up-regulated of pro-apoptosis gene *Bad* and *Bax* expression levels in U251 and U87MG cells *in vitro* (Figures 2C and 2D). These results suggest that paclitaxel can significantly induce apoptosis of glioblastoma cells *in vitro*.

Paclitaxel inhibits growth and proliferation of glioblastoma through MMP-9-meidated p38/JNK signaling pathway

As shown in Figures 3A and 3B, paclitaxel suppressed *MMP-9*, p38 and JNK expression as well as JNK phosphorylation levels in U251 and U87MG cells. We observed that *MMP-9* inhibitor could decrease p38, JNK expression and JNK phosphorylation (pJNK) levels in U251 and U87MG cells (Figures 3C and 3D). Results also showed that *MMP-9* overexpression inhibited paclitaxel-down-regulated (p*MMP-9*PX) expression levels of p38, JNK and pJNK in U251 and U87MG cells (Figures 3E and 3F). Importantly, paclitaxel inhibited growth and proliferation of U251 and U87MG cells was also abolished by *MMP-9* overexpression (Figures 3G and 3H). These results suggest that paclitaxel can inhibit growth and proliferation of glioblastoma through *MMP-9*-meidated p38/JNK signaling pathway.

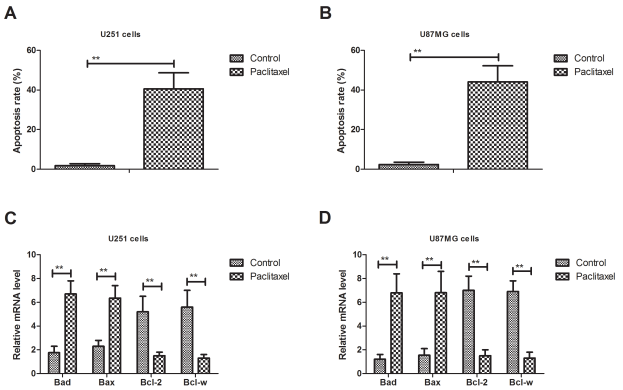

Figure 2. *Paclitaxel induces apoptosis of glioblastoma cells in vitro. (A, B) Paclitaxel (15 mg/ml) induces apoptosis of U251 (A) and U87MG (B) cells. (C, D) Paclitaxel down-regulates anti-apoptosis gene Bcl-2 and Bcl-w expression and up-regulates of pro-apoptosis gene Bad and Bax expression levels in U251 (C) and U87MG (D) cells.*

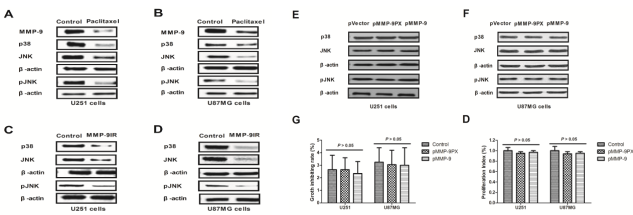

Figure 3. *Paclitaxel inhibits growth and proliferation of glioblastoma through MMP-9-meidated p38/JNK signaling pathway. (A, B) Paclitaxel suppresses MMP-9, p38 and JNK expression as well as pJNK levels in U251 (A) and U87MG (B) cells. (C, D) MMP-9 inhibitor decreases p38, JNK expression and pJNK levels in U251 (C) and U87MG (D) cells. (E, F) MMP-9 overexpression inhibits paclitaxel-down-regulated (pMMP-9PX) expression levels of p38, JNK and pJNK in U251 (E) and U87MG (F) cells. (G, H) MMP-9 overexpression blocks paclitaxel-inhibited growth and proliferation of U251 (G) and U87MG (H) cells.*

Paclitaxel inhibits glioblastoma tumor growth in tumor-bearing mice

We further investigated *in vivo* efficacy of paclitaxel in tumor-bearing mice. As shown in Figures 4A and 4B, paclitaxel suppressed tumor growth in U251- and U87MG-bearing mice. Results also showed that *MMP-9*, p38 and JNK expression levels were significantly down-regulated in tumor tissues after treatment with Paclitaxel (Figures 4C and 4D). These results indicate that Paclitaxel could inhibit glioblastoma tumor growth in tumor-bearing mice.

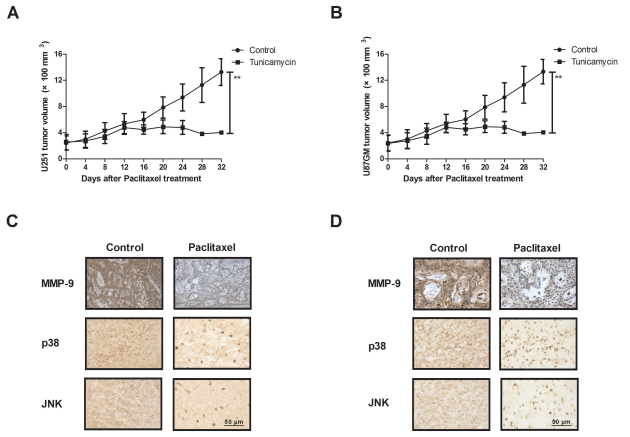

Figure 4. *Paclitaxel inhibits glioblastoma tumor growth in tumor-bearing mice. (A-B) Paclitaxel suppressed tumor growth in U251-bearing (A) and U87MG-bearing (B) mice. (C, D) Paclitaxel treatment down-regulates MMP-9, p38 and JNK expression levels in tumor tissues in U251-bearing (C) and U87MG-bearing (D) mice.*

Discussion

Glioblastoma exhibits a poor prognosis despite maximal multimodal therapy. Patients with an advanced glioblastoma are common accompanied with symptom of seizures and/or stroke which increased the difficulty and risk of clinical treatment [15]. Statistical review and meat-analysis has revealed that glioblastoma accounted for approximately 75% in all malignant tumors related the brain [16]. Evidences have indicated that paclitaxel presents anti-cancer potential for human malignant tumors through inducing apoptosis and inhibiting tumor cells growth and proliferation [17-19]. In this study, we investigated inhibitory effects of paclitaxel on glioblastoma cells. Findings indicate that paclitaxel markedly suppresses growth and proliferation as well promotes apoptosis of U251 and U87MG both *in vitro* and *in vivo*. Notably, we found that paclitaxel can inhibit growth and proliferation of glioblastoma through *MMP-9*-meidated p38/JNK signaling pathway.

Apoptosis sensitivity is crucial for the inhibition of glioblastoma growth [20,21]. Researches have showed that pro-apoptosis of *Bad* and *Bax* down-regulation enhance apoptotic resistance of glioblastoma cells [11,22]. Previous reports also suggest that anti-apoptosis of *Bcl-2* expression was down-regulated in glioblastoma cells that potential impact on the resistance of glioblastoma to temozolomide [23,24]. Additionally, *Bcl-w* enhances mesenchymal changes and invasiveness of glioblastoma cells by inducing nuclear accumulation of beta-catenin, which promotes growth and invasive potentials induced by *Bcl-w* down-regulation in glioblastoma multiforme [25,26]. In this study, our investigations revealed that paclitaxel treatment can significantly induce apoptosis of glioblastoma cells via inhibition of *Bcl-2* and *Bcl-w* expression, up-regulation of *Bad* and *Bax* expression levels *in vitro*. Involvement of apoptotic signaling pathways induced by paclitaxel contributes to inhibition of growth and proliferation of glioblastoma.

Currently, *MMP-9* mediates signal pathways have interested oncologists and clinical doctors for cancer research. Findings indicate that down-regulates the expression of *MMP-9* inhibits the growth and metastasis of tumor tissues [27]. *MMP-9* involves in Naringin-mediated glioblastoma cells growth through the inhibition of ERK-P38-JNK signaling pathway [28]. Inhibition of *MMP-9* gene expression in glioblastoma cell line through RNA interference leads to reduce tumor cells migration, invasion, growth and angiogenesis [29]. Interestingly, inhibitors p38 could arrest C6 glioblastoma cells and attenuate pro-inflammatory cytokine production and the invasiveness of human U251 [30,31]. Inhibition of JNK potentiates temozolomide-induced cytotoxicity in U87MG glioblastoma cells via suppression of AKT phosphorylation [32,33]. In this study, our results showed that Paclitaxel inhibits *MMP-9*, p38 and JNK expression in glioblastoma cells, suggesting *MMP-9* is a potential target for the treatment of glioblastoma.

In conclusion, findings in the current study have found that Paclitaxel inhibits growth and proliferation of glioblastoma cells both *in vitro* and *in vivo*. We found that Paclitaxel induces apoptosis of glioblastoma cells via regulation apoptosis-related gene expression. Notably, observations indicate that paclitaxel suppresses growth and proliferation through down-regulation of *MMP-9*-mediated p38/JNK signaling pathway in glioblastoma cells, which contributes to understand molecular mechanism medicated by Paclitaxel in the progression of glioblastoma.

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*Correspondence to

Feng Xu

Department of Neurosurgery

Xianning Central Hospital

The First Affiliated Hospital of Hubei University of Science and Technology

PR China