Int J Clin Exp Med 2019;12(1):652-657
www.ijcem.com /ISSN:1940-5901/IJCEM0080342

Original Article

Berberine inhibits cell growth via Wnt/beta-catenin signaling in glioma

Dajiang Xie*, Xinwei Li*, Xuchen Qi, Yingfeng Wan, Yinxin Zhu, Shuxu Yang

*Department of Neurosurgery, Sir Run Run Shaw Hospital, College of Medical Sciences, Zhejiang University, Hangzhou, China. *Co-first authors.*

Received May 26, 2018; Accepted November 10, 2018; Epub January 15, 2019; Published January 30, 2019

Abstract: Glioma is a common intracranial tumor, and the prognosis is poor, although much management is put into use. It has been reported that the Wnt/beta-catenin signaling pathway is associated with glioma. In the present study, berberine was investigated as an antineoplastic drug that inhibits Wnt/beta-catenin signaling pathway in growth of glioma cells *in vitro*. The results indicate that berberine inhibits cell proliferation and induces cell apoptosis. The expression levels of Wif-1 mRNA and protein in negative control (NC) cells were decreased significantly compared with berberine treated cells. Subsequent investigation revealed that berberine suppressed the activity of Wnt/beta-catenin signaling pathway and beta-catenin/TCF-4 transcription. Furthermore, expression of apoptosis-related protein Bcl-2 indicates cell apoptosis significantly. This study provides the first evidence that berberine reactivates Wif-1 from an inactive state and downregulates the canonical Wnt/beta-catenin signaling pathway. The findings identify berberine as an effective agent for glioma *in vitro* and may be a new therapeutic option *in vivo*.

Keywords: Berberine, Wif-1, Wnt/beta-catenin signaling, glioma

Introduction

Glioma is one of the most common malignant tumors in the central nervous system, with a median survival of 1 year, despite rapid progress, new insights, and new technology in the therapy and nursing care. Poor prognosis has remained a major issue during the past decades, in particular in the case of glioblastoma [1-5]. Therefore, it is essential to investigate the mechanism of the development and progression and explore new approaches for how to cure it.

Wnt/beta-catenin signaling pathway has been proven to be associated with various disease pathologies, especially in gliomagenesis. The pathway activates downstream targets and thereby regulates many biological processes through a complex of beta-catenin and T cell factor/lymphoid-enhancer factor 1 (TCF/LEF-1) family. Wnt stabilizes cytosolic beta-catenin, which then binds to TCF/LEF-1 in the nucleus and recruits transcription factors Brg1 and CREB-binding protein to initiate Wnt-targeted gene expression [6].

Wif-1 acts as an antagonist and inhibits Wnt signaling by direct binding to Wnt molecules. Wif-1 silencing, due to promoter hypermethylation, has been observed in tumor [7-9].

Berberine is an isoquinoline alkaloid isolated from roots, rhizomes, bark, and stems of members of the genus Berberis and Coptis. Berberis and Coptis used as drugs have a long history, such as antimicrobial agents to treat bacterial, fungal, and virus infections. During recent years, cardiovascular, and metabolic effects, growth-suppressive and anti-neoplastic activities of berberine were reported [10].

In this study, berberine suppressed the Wnt/beta-catenin signaling pathway via upregulation of Wif-1 in glioma cells. The results indicate that berberine blocks the Wnt/beta-catenin signaling pathway, with impaired nuclear translocation of beta-catenin. The results establish an important role for berberine in the treatment of

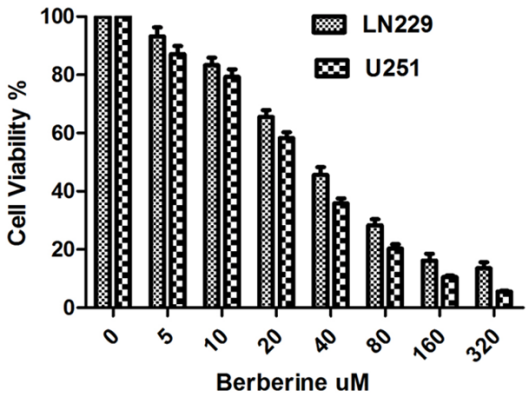

Figure 1. Proliferation effects of berberine in LN229 and U251 cells. Cells were treated with berberine (0-320 uM) and incubated for 24 hours and proliferation was assessed by MTS assay. Experiments were performed independently at least 3 times. Data are the mean ± SD.

brain gliomas, with potential use to lessen patients' suffering from gliomas.

Materials and methods

Cell culture, culture conditions and reagents

The human LN229 and U251 cell lines were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science. All cells were maintained in a 37°C, 5% carbon dioxide incubator in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal-bovine serum (Invitrogen). Berberine was purchased from Minglang Biotech Co., Ltd. and maintained in -20°C (Xian, PRC). Primary antibodies for Bcl2, Wif-1, Beta-catenin, and Beta-actin were purchased from Cell Signaling Technology (Beverly, Massachusetts, USA).

Cell proliferation assay/MTS assay

Cells were plated in 96-well plates at a density of 5000 cells/well. Cells were allowed to attach overnight in growth medium. After 24 hours, cells were treated with NCTD. After incubation for 72 hours, cellular proliferation was measured using the MTS assay and absorbance was measured at 490 nm. Proliferation data are presented as mean ± SD.

Apoptosis assay

Twenty-four hours after treatment, apoptosis in cultured cells was evaluated using annexin V labeling. For the annexin V assay, an annexin V-FITC labeled Apoptosis Detection Kit (Abcam) was used according to the manufacturer's protocol. Three independent experiments were performed and the data are presented as the mean + SD.

RNA extraction and quantitative real-time polymerase chain reaction

Total RNA was isolated using Trizol reagent (Invitrogen) and cDNA was synthesized using the High Capacity cDNA Archive kit (Applied Biosystems) according to the manufacturers' protocols.

Wif-1 and mRNA was quantified by qRT-PCR using SYBR Premix Ex Taq (Applied Takara Bio). Primers were as follows: Wif-1 were 5'-CCGAAATGGAGGCTTTTGTA-3' (forward) and 5'-TGGTTGAGCAGTTTGCTTTG-3' (reverse). PCR conditions included an initial denaturation step of 95°C for 2 minutes, followed by 35 cycles of 95°C for 10 seconds, 56°C for 20 seconds, and 72°C for 20 seconds, and a final elongation step of 72°C for 10 minutes. All qRT-PCRs were performed in duplicate. Quantitation relative to the endogenous control was done using the Applied Biosystems 7500 Fast System SDS software.

Western blotting

Extraction of proteins from cultured cells was followed by immunoblotting with the relevant antibodies. Each experiment was repeated at least three times.

Immunoflourescence staining assay

Immunofluorescence staining was conducted with LN229, U251 cells cultured on cover slips. The cells were fixed in 4% paraformaldehyde and permeabilized for 10 minutes in buffer containing 0.1% Triton X-100. The relevant antibodies were then added at the dilutions recommended by the manufacturers. DAPI reagent was used to stain the glioma cell nuclei, and the cells were visualized using FV-1000 laser-scanning confocal microscopes and analyzed using IPP5.1 (Olympus).

Statistical analysis

Unpaired Student's t-test was used to evaluate the statistical significance of *in vitro* and *in vivo*

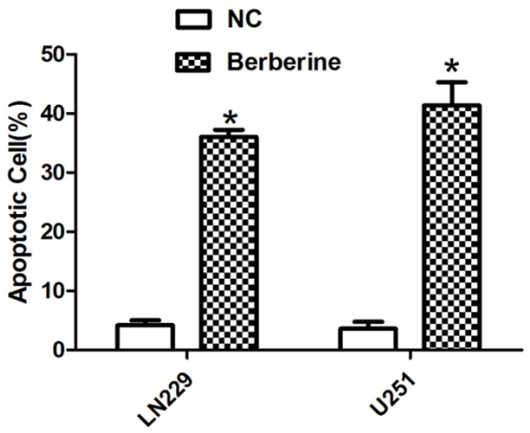

Figure 2. Berberine promotes apoptosis. Berberine significantly led to cell apoptosis in both LN229 and U251 cells (*P<0.05), relative to the NC.

treatments. All differences were considered to be statistically significant at the level of P<0.05. Statistics were performed using the SPSS Graduate Pack, version 11.0, statistical software (SPSS).

Results

Berberine suppressed growth of U251, LN229 cell lines

In the study, LN229 and U251 cells were exposed to 0, 5, 10, 20, 40, 80, 160, and 320 uM berberine for 48 hours. The results indicated that the cellular viability was decreased with the increase of the concentration of berberine (**Figure 1**). The 50% growth inhibition (IC50) of berberine was 40 uM for LN229 and 30 uM for U251 at 48 hour, respectively.

Berberine increased cell apoptosis in LN229 and U251 cell lines

Berberine also inhibited glioma cell survival. As shown in **Figure 2**, compared with NC group (4.30% ± 1.45% and 3.60% ± 1.10%) in LN229 and U251 cells, the treatment of berberine caused a significant increase (36.00% ± 1.73% and 41.35% ± 2.10%) in apoptotic death (P< 0.05). Bcl-2 protein level decreased in cell lines, as shown using Western blotting (**Figures 2** and **3A**).

Expression of mRNA transcript and protein expression of WIF-1 in U251, LN229 cells

RT-PCR assay was also performed to analyze the expression of Wif-1 in transcription level. Wif-1 transcript was down-regulated in U251 and LN229 cells. In contrast, it was up-regulated in berberine treated cells (**Figure 3C**). The results show that Wif-1 expression in U251 and LN229 NC cell lines (10.00 + 0.00) was significantly lower compared with berberine treated cells (35.50 + 3.80, 40.30 + 4.30) (P<0.05).

To detect the expression level of Wif-1, Western blotting was performed in U251 and LN229 cells (**Figure 3A** and **3B**). The level of WIF-1 expression was significantly lower in U251 and LN229 cells than that in berberine treated cells.

Berberine inhibits beta-catenin protein

Beta-catenin and Wnt-target proteins and their expression in many cell lines were descripted. Wnt signaling pathway has been proven to be associated with various disease pathologies. Berberine was reported merely to act as a negative regulator of Wnt signaling. Western blotting of total protein extracts from LN229 and U251 cells revealed that berberine reduced total beta-catenin content significantly (**Figure 3A** and **3B**). Immunofluorescence assays in the LN229 and U251 cells revealed nuclear location of beta-catenin. After the berberine treatment, beta-catenin was mainly located in the cytoplasm (**Figure 4**). In PBS-treated cells, beta-catenin was mainly located in the nucleus. The location of beta-catenin in cells shifted from the nucleus to the cytoplasm when the cells were treated with berberine.

Discussion

Glioma is the most frequent type of brain tumor and it is invariably associated with a poor prognosis. Standard treatment includes surgery, radiotherapy, and chemotherapy [1-4]. Prognosis is dismal with an average survival of approximately 12 months despite the rapid progress in new insights and technology in therapy and nursing care, the poor prognosis of patients with glioma has been remained during the past decades [2-5].

The mechanism of the development and progression depend on many factors and high-grade gliomas are heterogeneous tumors in both cytology and genetics signatures [5]. One major signaling pathway that has been linked to glioma is Wnt (wingless-type mouse mammary tumor virus integration site family).

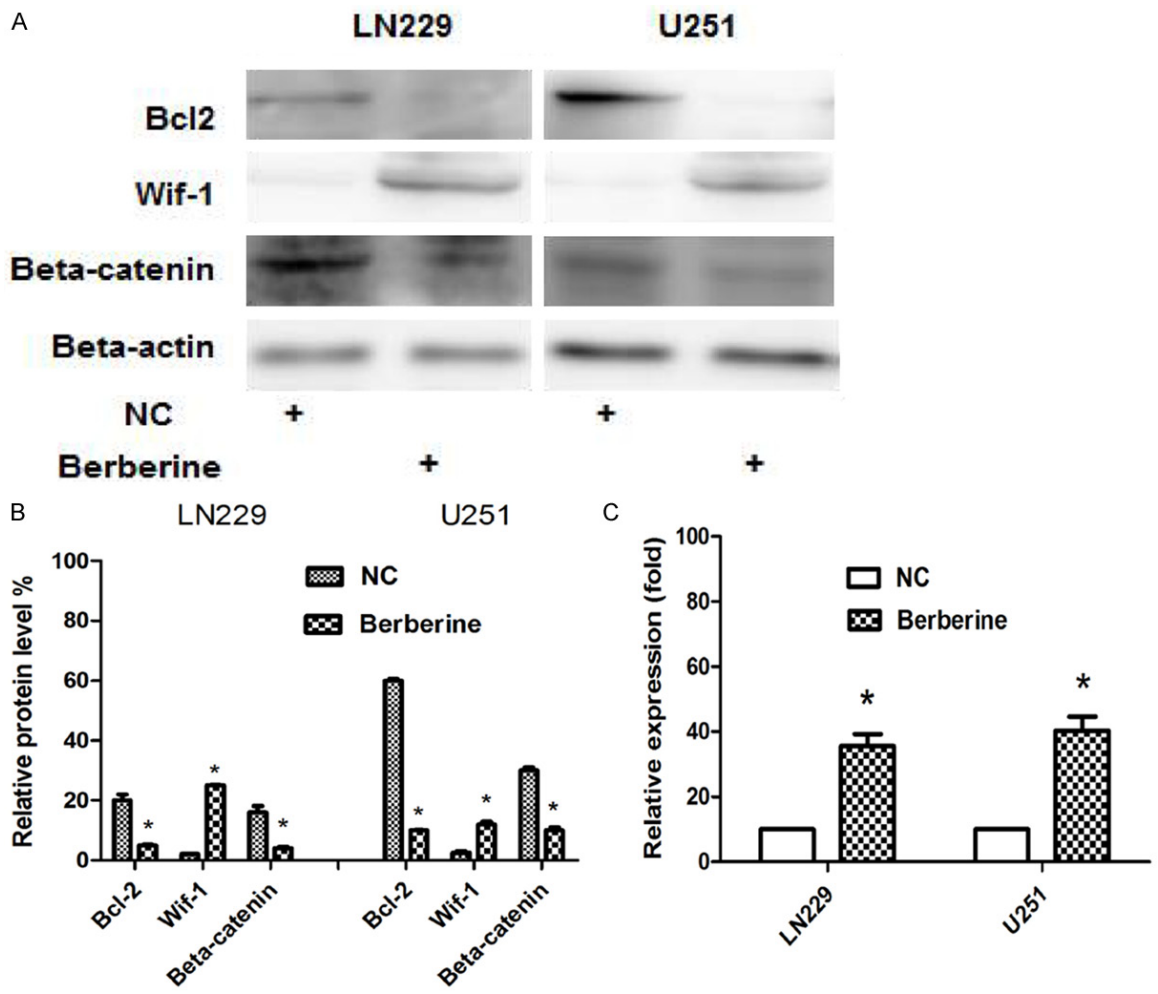

Figure 3. Expression of related protein and WIF-1. A and B. Expression of related protein changed in LN229 and U251 cells. Relative protein levels were determined with Western blot analysis using beta-actin as an internal control. C. WIF-1 mRNA expression was quantified by qRT-PCR analysis, and expression was promoted significantly in both LN229 and U251 cells (*P<0.05), relative to the NC.

The Wnt signaling pathway activates Wnt-downstream targets and thereby regulates many biological processes through a complex of beta-catenin and the T cell factor/lymphoid-enhancer factor 1 (TCF/LEF-1) family. Wnt stabilizes cytosolic beta-catenin, which then binds to TCF/LEF-1 in the nucleus and recruits transcription factors Brg1 and CREB-binding protein to initiate Wnt-targeted gene expression [6]. Wnt signaling has been proven to be associated with embryonic development, tissue renewal and regeneration, and various cancer pathologies, such as cell proliferation, cell cycle, cell death, and cell invasion/migration [6, 11-14].

The Wnt/beta-catenin signaling pathway is constitutive activated in glioma [15], aberrant expression of beta-catenin in astrocytic gliomas and glioblastoma is linked to a higher tumor grade [16]. Inhibitory Wnt pathway components in human cancers are inactivated by epigenetic events. As such, Wif-1 in high-grade gliomas is downregulated by promoter hypermethylation [7].

Berberine is an isoquinoline alkaloid isolated from roots, rhizomes, bark, and stems of members of the genus Berberis and Coptis. Berberis and Coptis used as drugs have a long history, such as antimicrobial agents to treat bacterial, fungal, and virus infections. During recent years, cardiovascular, and metabolic effects, growth-suppressive and anti-neoplastic activities of berberine were reported [17].

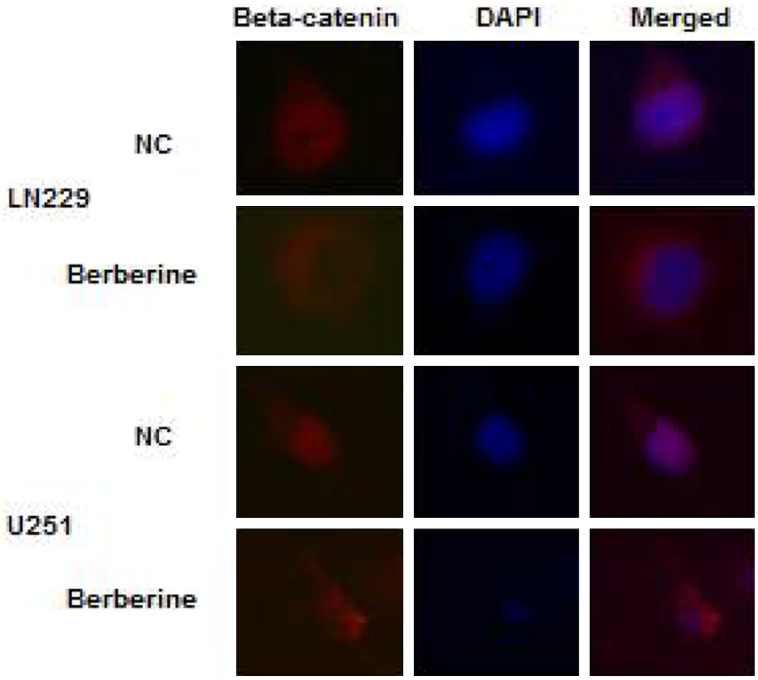

Figure 4. Immunofluorescence assay for beta-catenin. The location of beta-catenin in cells shifted from the nucleus to the cytoplasm when treated by berberine, compared with NC.

However, few study reported the anticancer effect of berberine against human glioma cells and early study of anti-glioma focused on Wnt/ beta-catenin signaling and nucleic acid interaction. Similarly, berberine can promote the loss of beta-catenin activation and inhibit the proliferation with dominant beta-catenin signaling [18]. These data suggest that berberine has significant therapeutic potential for the cancer via Wnt/ beta-catenin pathways.

Wif-1 silencing may be an early epigenetically carcinogenic event and plays a role in tumor development and progression [7-9]. Herein, we hypothesize that berberine may be used as an effective and nontoxic demethylating agents of the Wif-1 promoter. Aberrant methylation of promoter regions that silences transcription of the genes has been recognized as a mechanism for inactivating tumor suppressor genes.

In this study, untreated cells with down-regulated expression of Wif-1 showed a reversal after exposure to berberine. Berberine also suppressed nuclear translocation and expression of beta-catenin as measured using Western blot analysis. These results are consistent with our hypothesis that berberine may recover the Wif-1 expression level, and downregulate the Wnt canonical pathway in glioma cells.

Conclusions

The first evidence is provided that berberine reactivates Wif-1, downregulates the canonical Wnt pathway, and these studies establish an important role for berberine in the treatment of gliomas and a potential use to release patients' suffering.

Acknowledgements

This work was supported by Natural Science Foundation of Zhejiang Province of China (LY14H160025) and National Natural Science Foundation of China (81702974/ 81402044).

Disclosure of conflict of interest

None.

Abbreviations

NC, Negtive control; Wif-1, Wnt inhibitory factor-1.

Address correspondence to: Dr. Shuxu Yang, Department of Neurosurgery, Sir Run Run Shaw Hospital, College of Medical Sciences, Zhejiang University, Hangzhou 310016, China. Tel: +86-13819482125; Fax: +86-571-86044817; E-mail: 3195019@zju.edu.cn

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