Int J Clin Exp Med 2018;11(8):7848-7855
www.ijcem.com /ISSN:1940-5901/IJCEM0057001

Original Article

Antitumor effects of *Yi-Wei-Jie-Du* decoction (YWJD) against gastric carcinoma via BGC-803 tumor xenografts mice model

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Received May 8, 2017; Accepted September 15, 2017; Epub August 15, 2018; Published August 30, 2018

Abstract: To investigate the antitumor effects of *Yi-Wei-Jie-Du* decoction (YWJD) against gastric carcinoma. In this paper, we established a BGC-803 tumor xenografts mice model, then the tumor growth curves and tumor weight were analyzed. In addition, TUNEL assay and Ki67&CD34 staining were carried out. Quantitative Real-time fluorogenic PCR (qRT-PCR) and western blotting assays were performed to determine the levels of caspase-3, VEGF, PCNA, Cyclin D1, p-ERK 1/2, and ERK 1/2. Our present results showed that YWJD (100, 200 and 400 mg/kg/day) significantly inhibited both the tumor weight ($p < 0.01$) and growth ($p < 0.01$) of BGC-803 GC tumors, with a dose-dependent manner. Immunohistochemical results indicated that YWJD decreased the protein levels of Ki67 and CD34. TUNEL assay revealed that YWJD promoted the tumor cells apoptosis. Furthermore, our results also indicated that YWJD (100, 200 and 400 mg/kg/day) dose-dependently down-regulated the mRNA and protein expressions of PCNA ($p < 0.01$), VEGF ($p < 0.01$), Cyclin D1 ($p < 0.01$) and p-ERK ($p < 0.01$), whereas up-regulated the Caspase-3 ($p < 0.01$). Collectively, the present study suggested that YWJD has antitumor effect against GC, and the mechanisms might be involved in apoptosis and cycle arrest via down-regulating Ki67, CD34, PCNA, VEGF, Cyclin D1 and *p*-ERK whereas up-regulated C-caspase-3.

Keywords: Antitumor effects, *Yi-Wei-Jie-Du* decoction, gastric carcinoma, apoptosis, cycle arrest

Introduction

Increasing evidences have demonstrated that cancer, one of the leading causes of death, is a major public health threatening both in women and men in the world [1]. In particularly, the malignancy could not only cause high mortality, but also lead to huge money loss [1-3]. Gastric carcinoma (GC) is one of the most common malignant cancers with a high mortality, especially in some Southeast countries such as China, Japan and Korea [4-6]. Moreover, the diagnosis and treatment methods on GC have been currently improved a lot, but the prognosis and survival rate of GC is still poor [7]. So far, the synthetic chemical antit umor drugs, whichhave severe toxic effects, are commonly the only selective option for cancer chemotherapy besides surgery [8]. Therefore, it is urgent for medical scientist to find new effective therapeutic agents/strategies against GC. A growing number of investigations have revealed that traditional Chinese medicines (TCMs) and their formulas have notable potentials for treating/preventing cancers either being used alone or combining to synthetic chemical drugs [9, 10].

Yi-Wei-Jie-Du decoction (YWJD) is a reliable TCM formula used for treating GC in our hospital for decades with few side-effects. The YWJD is composited by *Ginseng Radix* (12 g), *Rhizoma Atractylodis* (15 g), *Wolfi poriaextensa* (15 g), *Curcuma Zedoaria* (12 g), *Radix Actinidiae chinensis* (30 g), *Spreading Hedyotis* Herb (30 g) and *Glycyrrhiza Uralensis* (6 g). However, so far, no report or study has reported the experimental pharmacological effects and possible molecular mechanisms of YWJD in laboratory for treating GC. Thus, in this reported paper, we investigated the antitumor activities of GC on a

Table 1. Primers used for quantitative real-time PCR in the present study

Gene		Sequence (5'-3')
PCNA	Forward	ATGAGCCTGTTCACCTAACG
	Reverse	GCAATGCCTAAGATGCTTCC
Cyclin D1	Forward	AAATGCCAGAGGCGGATGAG
	Reverse	TGGAGGGTGGGTTGGAAATG
VEGF	Forward	TCACCAAAGCCAGCACATAG
	Reverse	TTTCTCCGCTCTGAACAAGG
Caspase-3	Forward	ACTGGCGTGTGCGAGATGAG
	Reverse	CAGCAGCAGCAACAGCAGAC
GAPDH	Forward	AACAGCAACTCCCACTCTTC
	Reverse	TGGTCCAGGGTTTCTTACTC

BGC-803 cell tumor xenografts animal model and explored the potential molecules mechanisms, which could be helpful for developing the YWJD as a Chinese patent drug for treating GC in the future.

Materials and methods

Reagents and cell lines

BGC-803 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) at 37°C in 5% CO_2/95% air. All the crude Chinese materials in YWJD were obtained from the pharmaceutical preparation section of our hospital, and decocted with water for 2 times (each extraction was lasted for 1 h). Subsequently, the extracts were filtrated and dried with a freeze drier.

RPMI-1640 medium, Trizol reagents and FBS was purchased from the Invitrogen Inc., (Waltham, MA, USA); TUNEL detection kit was purchased from Roche Co. (Basel, Switzerland); quantitative real-time RT-PCR reaction kit (SYBR Green) was purchased from the Thermo Fisher Scientific (Waltham, MA, USA); PVDF membrane was purchased from EMD Millipore (Shanghai, China); Ki67 antibody for immunohistochemical analysis and primary antibodies of Cleaved-caspase-3 (C-caspase-3) and vascular endothelial growth factor (VEGF) for western blotting were purchased from the Abcam Biotechnology (Cambridge, MA, USA); primary antibodies of proliferating cell nuclear antigen (PCNA), Cyclin D1, p-ERK 1/2, ERK 1/2 and GAPDH were purchased from the Cell Signaling Technology (Beverly, MA, USA); BCA protein assay kit, goat-anti-rabbit (HRP) and ECL-detecting reagent were purchased from BeyotimeCo. (Jiangsu, China).

Xenograft model in nude mice

Total 30 BALB/C nude mice were purchased from the Shanghai laboratory animal center (Shanghai, China), and all our animal protocols were approved by the Animal Experimentation Ethics Committee of our hospital. In our present study, there are five experimental groups (each group was consisted by 6 nude mice, n = 6): Control group, Positive group (Sorafenib was used as the positive drug, 70 mg/kg) and three YWJD treatment groups (100, 200 and 400 mg/kg). All the animals were injected in the right flank subcutaneously with BGC-803 cells (3.0×10^6 per animal). When the tumors grew to approximate 3 mm in diameter, the mice were administered orally withsaline (10 ml/kg/day), positive drugs (70 mg/kg/day) and YWJD (100, 200 and 400 mg/kg/day), respectively. Then, the tumor sizes of nude mice were measured at 12, 15, 18, 21, 24, 27, 30 and 33 days by using a vernier caliper [tumor volumes = (width^2 × length)/2]. Additionally, the mice were sacrificed at the 33 days, and the tumor were collected and weighed. Then, the tumor tissues were stored for the following investigations.

Histopathological examinations

Tumor tissues were collected and fixed in 10% neutral formalin, and then embedded in paraffin. Subsequently, the tissues were sectioned to 5 μm thickness. After deparaffinization and rehydration, the sections were stained by TUNEL detection kit or Ki67 or CD34 antibody. The histopathological changes of the tumor tissues were observed under a microscope (OLYMPUS, Tokay, Japan).

Quantitative real-time fluorogenic PCR (qRT-PCR) assays

Tumor tissues were collected and homogenized, and total RNA was extracted byTrizol reagent. Then, total RNA was used for cDNA synthesisby reverse transcription using a qRT-PCR (ABI-7300, USA). All used mRNA primers (showed in **Table 1**) were designed by Premier 5.0 and synthesized by the JRDun Biotech. (Shanghai, China). Reverse transcription was

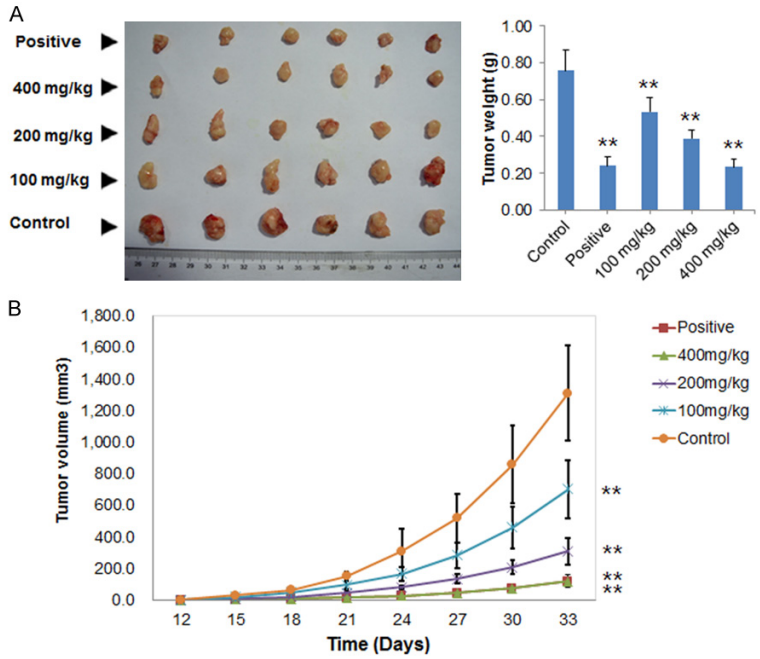

Figure 1. Antitumor effect of YWJD against GC *in vivo*. A. Tumor weight of the xenograft nude mice. B. Tumor growth curves of the xenograft nude mice. Data were expressed as mean ± SD (n = 6), and asterisk indicated significant difference, $^{**}p < 0.01$, compared with control.

carried outas the manufacturer's instructions of the SYBR Green quantitative real-time RT-PCR reaction kit. The relative mRNA expressions were analyzed by $2^{-\Delta\Delta CT}$ relative quantitative analysis in each sample.

Western blotting assay

Tumor tissues were homogenized and total proteins were extracted. After determination of the protein concentrations by the BCA Protein Assay Kits, an equal amount of protein (40 μg) was loaded toSDS-PAGE and subsequently blotted to PVDF. Then, the primary antibodies of PCNA, Cyclin D1, p-ERK 1/2, ERK 1/2, VEGF, C-caspase-3 and GAPDH were used to evaluate the corresponding proteins, followed by incubation with second antibody of HRP. Finally, the proteins' bands were visualized with an ECL-detecting reagent. To normalize the protein loading, GAPDH was used as the internal reference, and the proteins expression levels were expressed as a relative value to that of GAPDH.

Statistical analysis

Data were represented as mean ± SD and *P* value less than 0.05 was considered statistically significant. One way analysis of variance (ANOVA) was used to compare the means between two groups. Data analysis was performed using-SPSS software package (SPSS for Windows 19.0, SPSS Inc., IL, USA).

Results

YWJD showed significant inhibitory effect against GC in vivo

As shown in **Figure 1**, the antitumor effect of YWJD against GC was evaluated via a BGC-803 tumor-bearing nude mice model. Similar with the positive drug (70 mg/kg/day), the results showed that YWJD at the doses of 100, 200 and 400 mg/kg/day could significant inhibit both the tumor weight ($p < 0.01$) and growth ($p < 0.01$) of the BGC-803 GC tumors compared with the control mice, with a dose-dependent manner. In addition, the immunohistochemical results indicated that YWJD (100, 200 and 400 mg/kg/day) could decrease the protein levels of Ki67 and CD34 (**Figure 2**), which is another evidence for the antitumor effect of YWJD.

YWJD significantly promoted the GC tumor cell apoptosis

After demonstrating the antitumor effect of YWJD, the possible molecular mechanism of YWJD was explored. As can be seen from the **Figure 3**, TUNEL assay revealed that YWJD (100, 200 and 400 mg/kg/day) dependently promoted the tumor cells apoptosis, compared with the control mice.

YWJD down-regulated the expressions ofPCNA, VEGF and Cyclin D1 whereas up-regulated caspase-3

In addition, we also investigated the molecular mechanisms of YWJD based on the mRNA expressions of some genes related to the tumor growth. The results of the present investigation were showed in **Figure 4**. We can easily find

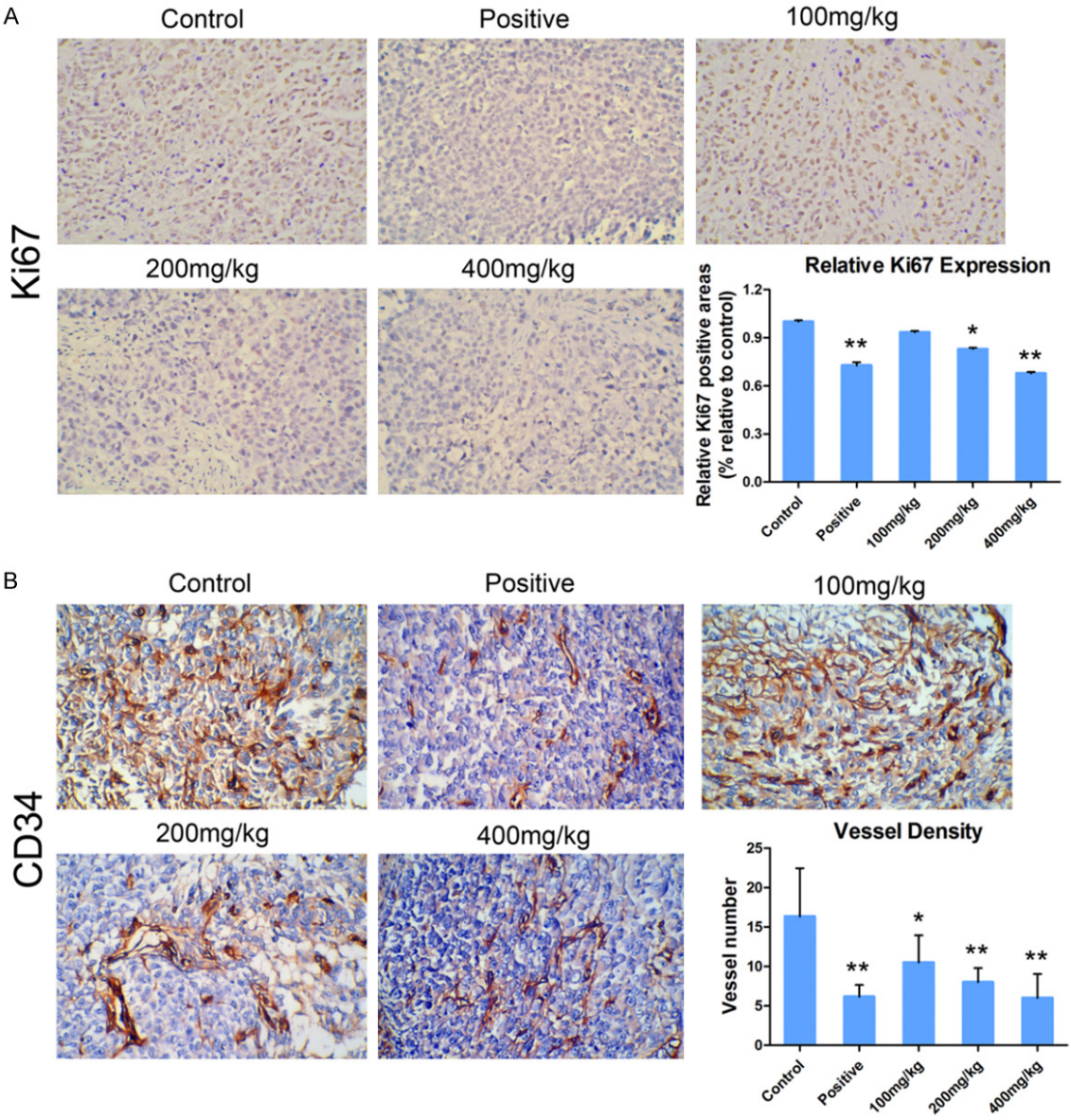

Figure 2. Results of the Ki67 and CD34 staining assay. A. Related Ki67 positive areas in five groups. B. CD34 positive areas and vessel density in five groups. Data were expressed as mean ± SD (n = 6), and asterisk indicated significant difference, $^{**}p < 0.01$, compared with control. Magnification, × 200.

that treatment with positive drugs could significant decrease the mRNA expressions of PCNA ($p < 0.01$), VEGF ($p < 0.01$) and Cyclin D1 ($p < 0.01$) whereas increase the mRNA expressions of caspase-3 ($p < 0.01$). In addition, similar with the positive drug, treating with YWJD (100, 200 and 400 mg/kg/day) could significantly down-regulated the mRNA expressions of PCNA ($p < 0.01$, $p < 0.01$, $p < 0.01$), VEGF ($p < 0.01$, $p < 0.01$, $p < 0.01$) and Cyclin D1 ($p < 0.01$, $p < 0.01$, $p < 0.01$), with an obvious dose-dependent manner. In contrary, the YWJD at the dose of 100, 200 and 400 mg/kg/day could notable dependently up-regulate the mRNA expressions of Caspase-3 ($p < 0.01$, $p < 0.01$, $p < 0.01$), compared with the control mice.

Additionally, we also studied the regulating effects of YWJD on proteins expressions of PCNA, VEGF, Cyclin D1 and C-caspase-3. As shown in **Figure 5**, a similar result to the mRNA was obtained. Our results demonstrated that YWJD (100, 200 and 400 mg/kg/day) could significantly down-regulate the PCNA ($p < 0.01$, $p <$

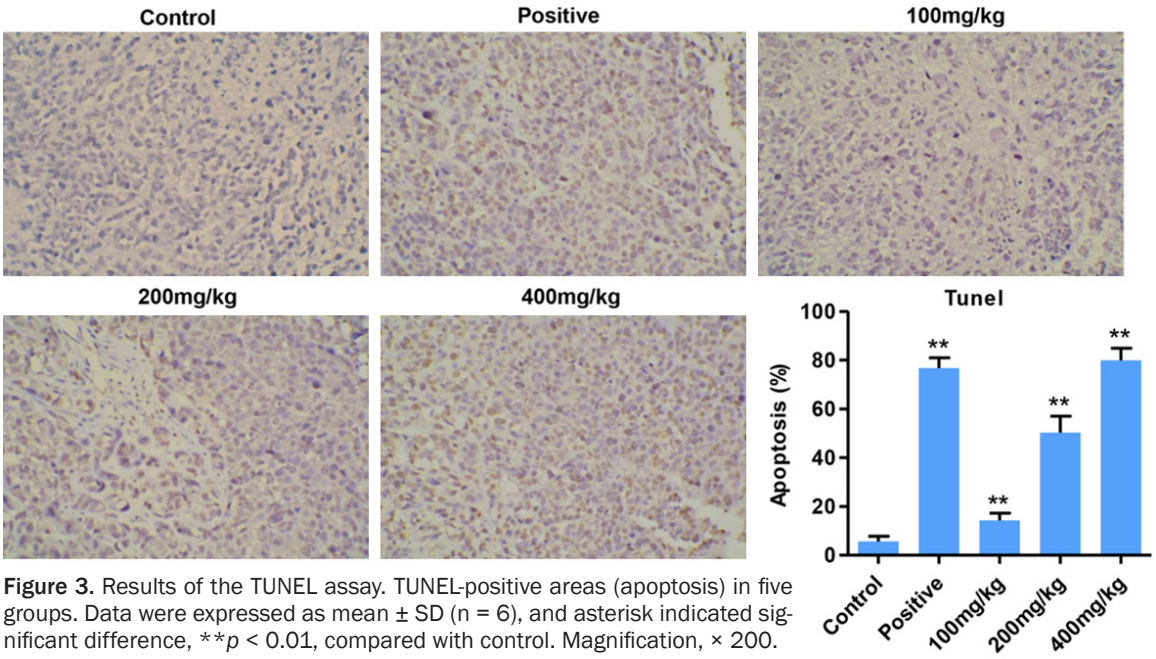

Figure 3. Results of the TUNEL assay. TUNEL-positive areas (apoptosis) in five groups. Data were expressed as mean ± SD (n = 6), and asterisk indicated significant difference, $**p < 0.01$, compared with control. Magnification, × 200.

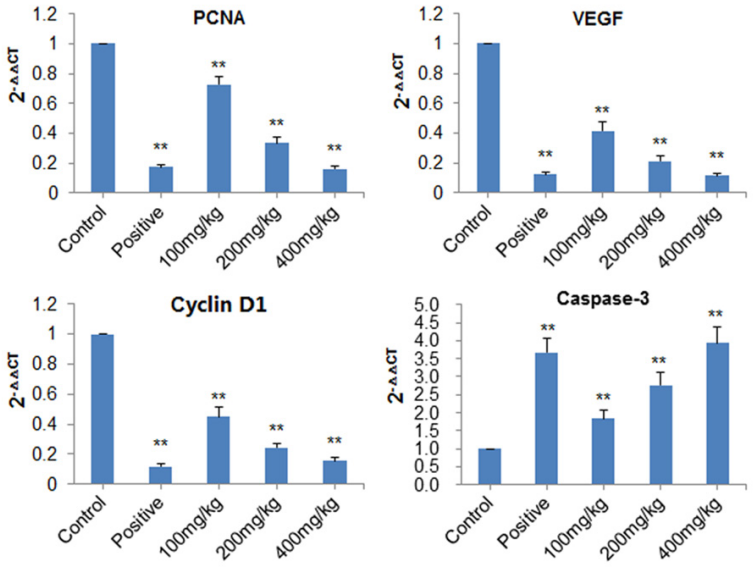

Figure 4. Results of the qRT-PCR assay. Data were expressed as mean ± SD (n = 6), and asterisk indicated significant difference, $**p < 0.01$, compared with control.

0.01, $p < 0.01$), VEGF ($p < 0.01$, $p < 0.01$, $p < 0.01$) and Cyclin D1 ($p < 0.01$, $p < 0.01$, $p < 0.01$), whereas up-regulate the C-caspase-3 ($p < 0.01$, $p < 0.01$, $p < 0.01$), compared with the control mice.

YWJD down-regulated the expressions of p-ERK 1/2

As can be seen from the **Figure 6**, results of the western blotting assay on ERK 1/2 and p-ERK 1/2 were shown. Our results showed that after treatment with YWJD, no obvious change could be observed for the expressions of ERK 1/2 ($p > 0.05$). Interestingly, YWJD (100, 200 and 400 mg/kg/day) could significantly decrease the p-ERK 1/2 in tumor tissues ($p < 0.01$, $p < 0.01$, $p < 0.01$), compared with the control mice.

Discussion

The formulas of TCMs have been used to treat diseases for thousands years, and are the essence of traditional Chinese medicinal theory [11, 12]. In our present investigation, we firstly reported the experimental evidence for demonstrating the antitumor effects of YWJD. In addition, we also findthat the potential mechanism is related to induction of apoptosis and cycle arrest.

In our present investigation, we evaluated the antitumor effect of YWJD with a xenografts nude mice model. In this present research, we demonstrated that YWJD could suppress the BGC-803 tumor's growth. Increasing researches have revealed that Ki67 is a useful biomark-

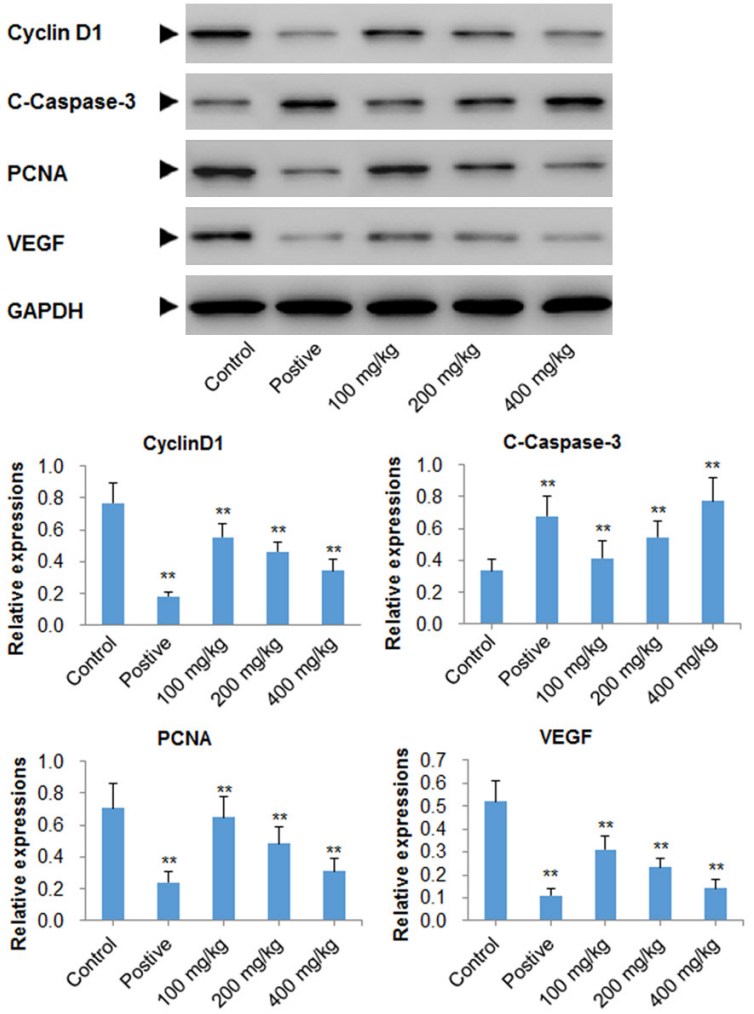

Figure 5. Results of the western blotting assay of Cyclin D1, C-caspase-3, PCNA and VEGF. Target proteins were expressed as relative expressions compared to the internal reference of GAPDH. Data were expressed as mean ± SD (n = 6), and asterisk indicated significant difference, $**p < 0.01$, compared with control.

er for evaluating the malignancy degree of GC in clinic, and Ki 67 is related to the GC cells' proliferation [13, 14]. Thus, down-regulation of the Ki67 expression might be useful for controlling the development of GC. Interestingly, from the histopathological examinations, wefound that YWJD treatment could notable decrease the Ki67 positive levels in tumor tissues. Modern researches have demonstrated that Cyclin D1 is a crucial protein for regulating the G1 phase in cell cycle. In addition, the over-expressed Cyclin D1 is closely correlated to the development of tumor. In clinic, Cyclin D1 has been comprehensively reported to be over-expressed in various tumor tissues, such as breast cancer, gastric cancer, lymphadenoma, and lung cancer, etc [15-17]. So, the over-expressed Cyclin D1 is also considered as a therapeutic target for treating cancers. Our results indicated that YWJD treatment significantly down-regulated the Cyclin D1 in tumor tissues of GC. PCNA is anecessary nuclear protein for the DNA synthesize of eukaryocyte, and its expression level in tumor tissues could reflect the cell proliferation. Previous reports have revealed that PCNA is also a potential biomarker for the growth degree of tumor [18, 19]. In our present study, the YWJD treatment could down-regulate the protein expression of PCNA, indicating that YWJD could inhibit the proliferation of BGC-803 cell. It's well known that apoptosis is the programmed physiological mode of cell death and is an idea way for treating cancers. In addition, caspase-3 protein is the most important mediator of cell apoptosis, and caspase-3 is also recognized as a crucial marker for cells undergoing apoptosis [20, 21]. Importantly, our results also demonstrated that YWJD could significantly up-regulate the caspase-3 in the tumor tissues. VEGF plays important roles in angiogenesis of tumor tissues, and angiogenesis is important for the growth and metastasis of tumor [22]. Therefore, decreasing VEGF expression in tumor tissues is a feasible way for controlling or treating tumor. Our experiment demonstrated that treatment with YWJD also down-regulated the VEGF expressions. What's more, CD34, a known endothelial marker of tumor vessel, is reported to be closely related to the angiogenesis of tumor tissues. Additionally, the CD34 is also considered as an important index for evaluating the malignancy and prognosis of GC [23-25]. The present immunohistochemical results showed that YWJD treatment could notable decrease the CD34 positive levels in tumor tissues. It's reported that ERK plays the key roles in trans-

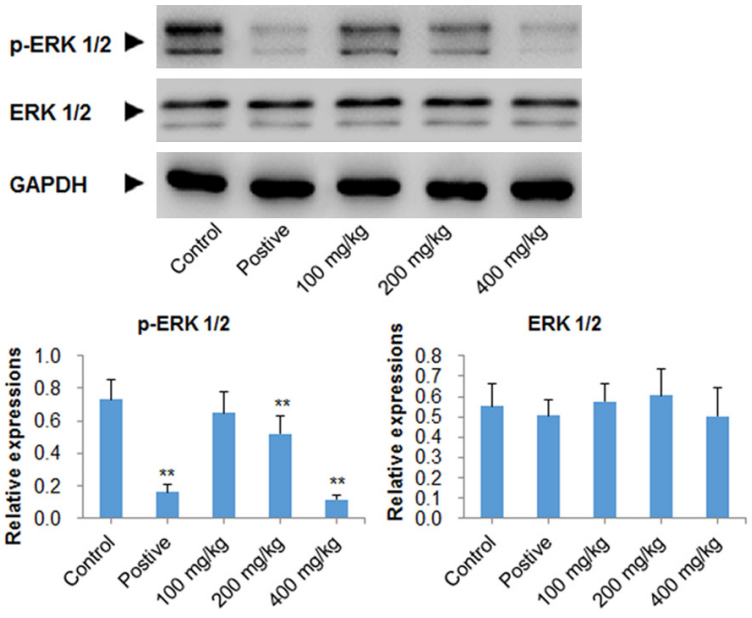

Figure 6. Results of the western blotting assay of ERK 1/2 and p-ERK 1/2. Target proteins were expressed as relative expressions compared to the internal reference of GAPDH. Data were expressed as mean ± SD (n = 6), and asterisk indicated significant difference, $**p < 0.01$, compared with control.

mission of cell signals to cell nucleus, and ERK could promote the growth, proliferation and apoptosis of tumorcell [26, 27]. Our results showed that YWJD treatment could decrease the expressions of phosphor-ERK 1/2 (p-ERK 1/2), indicating that YWJD could inhibit the proliferation of BGC-803 tumor cells.

In conclusion, our research demonstrated that YWJD possess antitumor effect against GC, and the mechanisms is involved in induction of apoptosis and cycle arrest via down-regulating PCNA, VEGF, Cyclin D1 and p-ERK whereas up-regulated C-caspase-3.

Disclosure of conflict of interest

None.

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