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Original Research Article

Zhao's Weitiao No 3 inhibits tumor growth in gastric cancer-bearing nude mice

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Abstract

Purpose: *To investigate the effect of Zhao's Weitiao No. 3 (ZW3) on the progression of gastric cancer (GC) in mice, and to determine its effects on the proliferation and apoptosis of gastric cancer tissues.*
Methods: *Tumor growth in vivo was used to determine the effect of ZW3 (15, 30, and 60 mg/kg) on GC growth in mice. Immunohistochemical and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assays were conducted to evaluate the effects of ZW3 on the proliferation and apoptosis of mouse GC tissues, while quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and immunoblot assays were conducted to determine its effects on GC cell apoptosis.*
Results: *ZW3 inhibited tumor growth in tumor-bearing mice, suppressed the expression of proliferation-related proteins ($p < 0.001$), increased the apoptosis of tumor cells in tumor-bearing mice ($p < 0.001$), and suppressed the expression of apoptosis-related proteins in mouse tumor tissues ($p < 0.001$).*
Conclusion: *Thus, ZW3 is a potential drug for the management of GC. However, further investigations are required to determine its full potential.*

Keywords: *Gastric cancer, Zhao's Weitiao No. 3 (ZW3), Proliferation, Tumor growth, Apoptosis*

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INTRODUCTION

Gastric cancer (GC) is the fifth most common cancer [1]. It is estimated that the 5-year survival of GC patients is < 20 % [2,3]. In the process of GC growth, biological processes such as tumor cell proliferation are significantly enhanced [4,5]. To determine whether drugs affect the biological behaviors of tumor cells by regulating the expressions of proliferation-related molecules, it is necessary to study the expressions of related molecules, such as Ki67, Proliferating cell nuclear antigen (PCNA), and Bcl-2 [6], and to improve the prognosis of GC, more promising drugs are needed.

Zhao's Weitiao No. 3 (ZW3) is mainly comprised of ludangshen, fried *Atractylodes atractylodes*, *Poria coix* seed, *Pinellia ginger*, tangerine peel, *Poria piriformis*, Huaishan medicine, wheat bud, malt, fu Shen, loquat leaves, and prepared glycyrrhiza. Its efficacy lies in fine-tuning the imbalance, fine-tuning the basic treatment of the spleen and stomach, indirect regulation of kidney qi and blood, mobilization of immunity, and restoration of its function. Balancing Yin, Yang,

qi, and blood results in effective tumor treatment [7]. Some clinical trials on ZW-3 have found that it improved the main clinical symptoms of colon cancer patients, and improved their quality of life [8].

Proliferating cell nuclear antigen is a helper protein of DNA polymerase, while Ki-67 is a molecule closely related to chromatin function. PCNA and Ki-67 are used as markers of cell proliferation. Bcl-2, Bax, and caspase-3 are key molecules that regulate mitochondrial apoptosis [9]. Bax and Bcl can form isodimer-2 to antagonize the anti-apoptotic effect of Bcl-2 and promote cell apoptosis [9]. In this study, the effect of ZW3 on the progression of GC cells was therefore studied.

EXPERIMENTAL

Antibodies and drugs

Anti-Ki67 antibody (1:200 dilutions for immunohistochemistry (IHC), ab15580), anti-PCNA antibody (1:200 dilutions for IHC, ab92552), anti-Bax antibody (1:500 dilutions, ab32503), anti-Bcl-2 antibody (1:500 dilutions, ab182858), anti-cleaved-caspase-3 antibody (1:1000 dilutions, ab32042), and anti-β-actin antibody (1:3000 dilutions, ab8226) were from Abcam (Cambridge, UK). The ZW-3 (15, 30, 60 mg/kg) was kept in the lab of Wuxi Affiliated Hospital of Nanjing University of Chinese medicine.

Immunohistochemistry (IHC) and TUNEL assays

The expressions of proteins in tissues were determined using IHC assays. The sections of mice tumors were fixed using 4 % paraformaldehyde (PFA) for 30 min and blocked with 2 % bovine serum albumin. The sections of mice tumors were subsequently incubated with the antibodies for 2 h at room temperature and secondary antibodies for 1 h at room temperature. TUNEL staining was conducted using the Dead End™ Fluorometric TUNEL System Kit (Promega, Madison, WI, USA).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After synthesizing cDNA using a reverse transcription kit, quantitative PCR was performed using an SYBR mixture (Takara, Shiga, Japan) using the following thermocycling conditions: initial denaturation at 95 ºC for 3 min, followed by 30 cycles of denaturation at 95 ºC for 30 s, annealing at 58 ºC for 30 s, extension at 72 ºC for 30 s, and a final extension at 4 ºC for 30 min. The relative mRNA levels were normalized to GAPDH. The qPCR primer sequences used are shown in Table 1.

Table 1: Quantitative PCR primer sequences

Gene	Forward sequence (5´-3´)	Reverse sequence (5´-3´)
Bax	AGACAGGGGCCTTTTTGCTAC	AATTCGCCGGAGACACTCG
Bcl-2	GCTACCGTCGTGACTTCGC	CCCCACCGAACTCAAAGAAGG
Caspase-3	CTCGCTCTGGTACGGATGTG	TCCCATAAATGACCCCTTCATCA
GAPDH	TCAACGGCACAGTCAAGG	TGAGCCCTTCCACGATG

Immunoblot assay

All samples were used to extract the total proteins by RIPA buffer (Beyotime, China) and separated using SDS-PAGE followed by transfer onto PVDF membranes, and blocked using 5 % milk. All PVDF membranes were incubated with primary antibodies for 2 h followed by secondary antibodies for another 1 h. Blots were quantified using an ECL kit.

Assessment of tumor growth *in vivo*

The study, which involved tumor growth *in vivo,* was approved by the Ethics Committee of Wuxi Affiliated Hospital of Nanjing University of Chinese medicine (approval no. 2021SJ004) and conducted in line with the National Institute of Health Laboratory Animal Care and Use Guidelines [10]. Female BALB/c nude mice (20 − 24 g, 8 weeks old) were obtained from Vital River Laboratories (Beijing, China). Ten nude mice were used (five for each group). Cells were injected into nude mice, and the mice were fed with ZW-3 (15, 30, and 60 mg/kg mice weight) every day. The tumor volume was measured every 6 days. After 30 days, the tumors were analyzed, and volumes were compared between the control and drug treatment groups.

Statistical analysis

GraphPad 6.0 (GraphPad, San Diego, CA, USA) was used for statistical analysis. Data are presented as the mean ± standard deviation (SD). Student's *t*-test was used for comparing the different groups. $P < 0.05$ were considered significant.

RESULTS

ZW-3 suppressed tumor growth

The ZW-3 (15, 30, and 60 mg/kg mice weight) was fed to mice every day. It inhibited GC growth (Figure 1), and both tumor volume and weight were significantly decreased upon ZW-3 feeding.

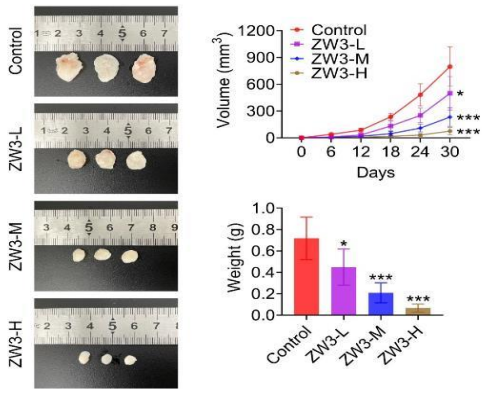

Figure 1: ZW-3 suppressed tumor growth of gastric cancer cells in mice. *P < 0.05, ***p < 0.001, ZW3-L, ZW3-M, and ZW3-H, versus the control

ZW-3 suppressed the expression of proliferation-related proteins

Because ZW-3 suppressed tumor growth of GC cells in mice, the expression levels of proliferation-related proteins such as Ki67 and PCNA were determined in tumor tissues from control or ZW-3–fed mice. Using IHC assays, it was found that the expressions of Ki67 and PCNA were decreased in ZW-3-treated tumor tissues, suggesting inhibition of cell proliferation (Figure 2). Therefore, ARHGEF19 stimulated breast cancer growth in mice, and ZW-3 suppressed the expression of proliferation-related proteins in mouse tumor tissues. Immunohistochemical assays showed the expressions of Ki67 and PCNA in tumor tissues from the mice with the indicated feedings.

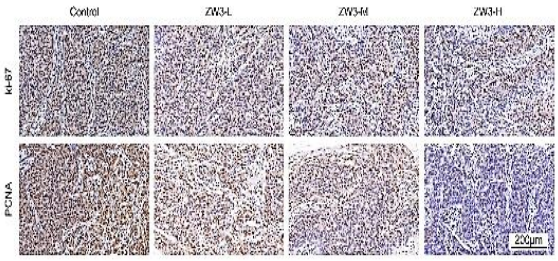

Figure 2: ZW-3 suppressed the expression of proliferation-related proteins in mouse tumor tissues. Scale: 200 μm

ZW-3 enhanced the apoptosis of GC cells

Figure 3 shows that ZW-3 treatment increased the apoptosis of GC cells in tumor tissues. The TUNEL assays showed the degree of apoptosis in mouse tumor tissues with the indicated feedings.

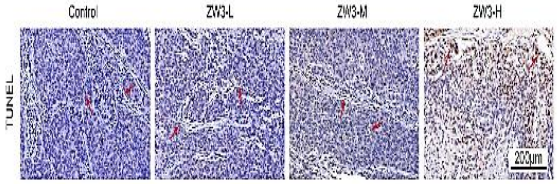

Figure 3: ZW-3 promoted the apoptosis of gastric cancer cells in mouse tumor tissues. Scale bar: 200 μm

ZW-3 mediated the expression of apoptosis-related proteins

The ZW-3 increased the expressions of Bax and cleaved caspase-3, but decreased the expression of Bcl-2, in tumor tissues (Figure 4 A). Similarly, data from immunoblot assays indicated that ZW-3 increased the expressions of Bax and cleaved caspase-3, and decreased Bcl-2 expression in tumor tissues (Figure 4 B). the ZW-3, therefore, increased apoptosis of GC cells in mouse tumor tissues.

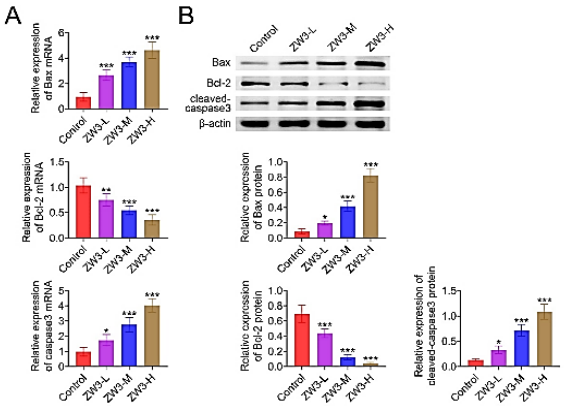

Figure 4: ZW-3 mediated expressions of apoptosis-related proteins in gastric cancer cells of mice. (A) Quantitative PCR assays showed mRNA levels of Bax, Bcl-2, and cleaved caspase-3 in tumor tissues from control, ZW3-L, ZW3-M, and ZW3-H groups. (B) Protein levels of Bax, Bcl-2, and cleaved caspase-3 in tumor tissues from control, ZW3-L, ZW3-M, and ZW3-H groups. *P < 0.05, **p < 0.01, ***p < 0.001, ZW3-L, ZW3-M, and ZW3-H versus control

DISCUSSION

Gastric cancer is a malignant tumor that occurs in the digestive tract [9]. Based on GLOBOCAN

statistics, there were about 951,600 new cases of GC worldwide in 2012, and 723,100 patients died of GC, with more than 70 % of the new cases from developing countries [11]. There were 405,000 new cases in China, accounting for 42.59 % of the global incidence of GC [12]. To improve the prognosis and survival of patients, it is therefore necessary to further elucidate the pathogenesis and develop therapeutic drugs [13]. Furthermore, because of poor early diagnosis of GC, most patients are diagnosed at advanced stages [14,15].

Chemotherapy is the main treatment for advanced GC. However, chemotherapy has limited effectiveness due to drug resistance and side effects [16]. Traditional Chinese medicine therefore may have potential value in GC treatment. In this study, ZW-3 had a significant inhibitory effect on GC in a mouse model, and could therefore serve as a potential drug for the treatment of GC. Traditional Chinese medicine has the characteristics of multiple targets and this is key in the treatment of GC, including regulating the body's immune function and improving surgery, radiation, and chemotherapy tolerance. The TCM also improves preoperative physical conditions, reduces radiation and chemotherapy side effects, improves clinical symptoms, and prevents and treats tumor recurrence [17]. The ZW-3 suppressed the proliferation and stimulated GC cell apoptosis in mice. In the present study, the regulatory effects of ZW-3 on the expressions of Ki67, PCNA, Bcl-2, Bax, and caspase-3 in GC were determined. The PCNA is a helper protein of DNA polymerase [6]. The Ki-67 is a molecule closely related to chromatin function, while, Bcl-2, Bax, and caspase-3 are key molecules that regulate mitochondrial apoptosis [9]. The Bcl-2 protein can regulate the opening and closing of mitochondrial membrane pores [9]. In addition, Bax protein and Bcl form isodimer-2 to antagonize the anti-apoptotic effect of Bcl-2 [18].

The Zhao Jingfang fine-tuning node balance theory was developed by Zhao Jingfang, based on 30 years of clinical practice. The theory hypothesizes that tumors arise from the patient's cells. The effects of ZW-3 treatments were assessed on GC progression and related molecules and regulatory genes, providing a solid foundation for the clinical application of ZW-3 in GC patients.

CONCLUSION

Zhao's Weitiao No. 3 inhibits tumor growth in GC-bearing nude mice by mediating proliferation and apoptosis. Thus, ZW3 is a potential drug for the treatment of GC, but further studies are required to confirm its efficacy.

DECLARATIONS

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Funding

None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was conducted by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Qi Pan and Pei Xiang designed and conducted the experiments. Yuanyuan Chen and Jingfang Zhao analyzed and interpreted the data, and prepared the manuscript for publication.

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