Original Article

Herbal Medicine Teng-Long-Bu-Zhong-Tang Inhibits the Growth of Human RKO Colorectal Cancer by Regulating Apoptosis, Senescence, and Angiogenesis

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Abstract

Background: Teng-Long-Bu-Zhong-Tang (TLBZT) is a traditional Chinese herbal medicine used to treat colorectal cancer (CRC). In the present study, we observed the anti-cancer effects of TLBZT on human RKO CRC. **Materials and Methods:** Mice were subcutaneously transplanted with RKO cells, divided into control, 5-Fu-administered, TLBZT-administered, and TLBZT and 5-Fu combination-administered groups, and treated with 5-Fluorouracil (5-Fu) and/or TLBZT. Apoptosis was detected by TdT-mediated dUTP nick-end labeling assay. The activity of caspase-3,-8, and-9 was detected using specific commercial kits. Cell senescence was assessed using senescence β-galactosidase staining. Protein expression was evaluated by immunohistochemistry. **Results:** TLBZT inhibited RKO CRC tumor growth, enhanced the anti-cancer effects of 5-Fu, induced apoptosis, and activated caspase-3,-8, and-9. TLBZT induced cell senescence accompanied by the downregulation of cyclin E1 and cyclin-dependent kinase 2 expressions. TLBZT also inhibited angiogenesis and the expression of hypoxia-inducible factor 1 subunit alpha and vascular endothelial growth factor-A. **Conclusions:** TLBZT inhibited RKO CRC tumor growth and enhanced the anti-cancer effects of 5-Fu, and it could be associated with apoptosis and cell senescence induction, and angiogenesis inhibition.

Keywords: Angiogenesis; apoptosis; cell senescence; Chinese herbal medicine; colorectal cancer

Introduction

Colorectal carcinoma (CRC) is a common malignancy. With the development of chemotherapy and targeted therapy, relatively satisfactory clinical efficacy for treating early and middle-stage CRC has been achieved. However, even in combination with targeted therapy, such as cetuximab, bevacizumab, regorafenib, and TAS-102, adequate efficacy for advanced CRC cannot be achieved.[1]

Traditional Chinese medicine (TCM) can help ameliorate clinical symptoms, improve immune function and quality of life, alleviate the toxic and side effects of chemotherapy and targeted therapy, and inhibit CRC tumor growth.[2-4] Based on TCM theory, clinical practice, and related studies, we have developed an herbal formula, Teng-Long-Bu-Zhong-Tang (TLBZT), for CRC treatment.

In combination with chemotherapy, TLBZT inhibits tumor growth in patients with advanced CRC.[5] TLBZT inhibits cell growth, induces apoptosis via the activation of caspases, and promotes cell senescence by regulating p16/p21-RB signaling in human cancer cells *in vitro*.[6-8] TLBZT inhibits tumor growth in animal models implanted with murine CT26 CRC cells.[9] TLBZT also inhibits lung metastasis in mouse models implanted with human RKO CRC cells.[10] In the present study, we further observed the anti-cancer effects of TLBZT on human RKO CRC.

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Materials and Methods

Chemicals and reagents

Trypsin and Dulbecco's modified Eagle's medium (DMEM)

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were purchased from Thermo Fisher (Waltham, MA, USA). Fetal bovine serum (FBS) was obtained from SAFC Biosciences (Lenexa, KS, USA). TdT-mediated dUTP nick end labeling (TUNEL) assay kit was purchased from Promega (Madison, WI, USA). 5-Fluorouracil (5-Fu) was procured from Xudong Haipu Pharmaceutical (Shanghai, China). Antibodies against cyclin-dependent kinase (CDK) 2 and cyclin E1 (CCNE1) were purchased from Bioworld Technology (St. Louis Park, MN). Antibodies against vascular endothelial growth factor A (VEGFA) and hypoxia-inducible factor 1 subunit alpha (HIF-1α) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-CD31 antibody was purchased from Abcam (Cambridge, MA, USA). Caspase-3, -8, and -9 assay kits were obtained from Beyotime (Haimen, Jiangsu, China). TLBZT compositions and their gas chromatography-mass spectrometry profiles have been described previously.[6-9]

Cell culture

Human CRC RKO cells were obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM (10% FBS and 1% Pen-Strep) in a humidified incubator with 5% CO_2 at 37°C. Logarithmically grown RKO cells were used in the animal experiments.

Animal models and treatment

Male BALB/c nude mice, aged 6–7 weeks, were obtained from the Shanghai SLAC Laboratory Animal Center and housed in the specific pathogen-free animal center of Longhua Hospital. RKO cells (5×10^6) were subcutaneously transplanted into the backs of mice. When the tumor grew to a measurable size (approximately 65 mm^3), the mice were randomly divided into control, 5-Fu-treated, TLBZT-treated, and TLBZT and 5-Fu combination (TLBZT +5-Fu)-treated groups and intragastrically administered 0.3 mL sterile water or TLBZT (1.5 g/mL, equivalent to crude herb materials) once a day and/or intraperitoneally injected with 0.2 mL 5-Fu (5 mg/mL) once a week. Tumor length (L) and width (W) were measured using calipers every 3 days. The tumor volume was calculated as follows: Volume = $\pi/6 \times L \times W^2$. All procedures and experiments involving mice were approved by the Animal Care and Use Committee of Longhua Hospital.

TdT-mediated dUTP nick end labeling assay

Apoptosis was detected using a TUNEL assay kit according to the manufacturer's protocol. Briefly, the tumor tissues were embedded in paraffin, sectioned, deparaffinized with xylene, dehydrated with gradient alcohol, and incubated with protease K for 20 min at room temperature. Next, the sections were washed with PBS and incubated with TdT reaction mix for 30 min at room temperature, and this reaction was terminated by using 2× SSC, followed by washing with PBS. The sections were then incubated with horseradish peroxidase-conjugated streptavidin for 30 min at 37°C, washed with PBS, developed using 3,3N-diaminobenzidine (DAB), counterstained with hematoxylin, and mounted with resin. Then, the sections were observed and photographed under a microscope and analyzed using Image-Pro Plus 6.0 software.

Caspase activity assay

Caspase activity was detected using caspase assay kits according to the manufacturer's protocol. Briefly, the tumor tissues were lysed, centrifuged at 12,000 rpm at 4°C for 15 min, and the supernatant was collected and quantified. The activity of caspase-3, -8, and -9 was detected using Ac-DEVD-pNA, Ac-IETD-pNA, and Ac-LEHD-pNA, as substrates, respectively.

Senescence β-galactosidase staining

Tumor tissues were frozen, sectioned, fixed with 2% glutaraldehyde and 2% formaldehyde for 10 min at room temperature, washed with PBS, and stained with X-gal solution overnight at 37°C.[11] Senescence β-galactosidase (SA-β-gal) staining was observed and photographed under a microscope and analyzed using Image-Pro Plus 6.0 software.

Immunohistochemistry

Paraffin-embedded tumor tissues were cut into 5-micrometer thick sections; deparaffinized with xylene; dehydrated by gradient alcohol; incubated in 3% H_2O_2 for 15 min at room temperature; blocked with 5% normal goat serum; incubated with antibodies against CD31 (1:50), CCNE1, CDK2, HIF-1α, and VEGFA (1:100) overnight at 4°C; washed with PBS; probed with secondary antibody for 30 min; developed using DAB; counterstained with hematoxylin; and mounted with resin. The sections were observed and photographed under a microscope and analyzed using Image-Pro Plus 6.0 software.

Statistical analysis

Data are expressed as the mean ± standard deviation and analyzed using one-way analysis of variance. $P < 0.05$ was considered statistically significant.

Results

Teng-Long-Bu-Zhong-Tang inhibited tumor growth

The effect of TLBZT on tumor growth was observed. RKO cells were subcutaneously implanted into BALB/c nude mice. TLBZT and/or 5-Fu were administered after tumor formation. The results showed that the RKO CRC tumor progressively grew, but TLBZT and 5-Fu inhibited RKO CRC cell growth ($P < 0.01$). TLBZT + 5-Fu treatment improved the anti-cancer effect on tumor growth compared with either treatment alone ($P < 0.01$) [Figure 1].

Teng-Long-Bu-Zhong-Tang promoted apoptosis

TUNEL assay was used to detect the effect of the drugs on RKO CRC cell apoptosis. The results showed that 5-Fu and TLBZT promoted apoptosis in RKO CRC tissues ($P < 0.01$), and TLBZT + 5-Fu treatment showed stronger effects than treatment with 5-Fu or TLBZT alone ($P < 0.01$) [Figure 2].

Teng-Long-Bu-Zhong-Tang activated the caspases

Apoptosis is an important anti-tumor mechanism mediated by the caspase cascade.[12] Thus, we also detected caspase activity.

The results showed that TLBZT and 5-Fu increased the activities of caspase-3,-8, and-9 in RKO CRC tissues ($P < 0.01$), and TLBZT + 5-Fu treatment showed stronger effects than 5-Fu or TLBZT alone ($P < 0.05$) [Figure 3].

Teng-Long-Bu-Zhong-Tang induced cell senescence

Cell senescence involves irreversible withdrawal from the cell cycle, and senescent cells no longer proliferate, which is an important anti-tumor mechanism.[13,14] SA-β-Gal staining is a classic method for detecting cell senescence.[11] In the present study, SA-β-Gal staining showed that TLBZT-induced cell senescence ($P < 0.01$) [Figure 4]. However, 5-Fu had no significant effect on RKO CRC cell senescence (data not shown).

Teng-Long-Bu-Zhong-Tang inhibited CDK2 and CCNE1 expression

Senescent cells exhibit an arrested cell cycle. We detected CDK2 and CCNE1 expression, both of which are essential for G1/S progression,[15] by immunohistochemistry (IHC). The results demonstrated that TLBZT inhibited CDK2 and CCNE1 expression ($P < 0.01$) [Figure 5].

Teng-Long-Bu-Zhong-Tang inhibited angiogenesis

Solanum nigrum L. and *Scutellaria barbata* D. Don are known to inhibit angiogenesis in different tumors.[16-18] Since TLBZT contains *S. nigrum* L. and *S. barbata* D. Don, we hypothesized that it could inhibit angiogenesis. IHC was used to detect angiogenesis in RKO CRC tissues using CD31 as a marker. As shown in Figure 6, TLBZT significantly inhibited angiogenesis in RKO CRC tissues compared with the control ($P < 0.01$). 5-Fu showed no significant effects on angiogenesis in RKO CRC tissues (data not shown).

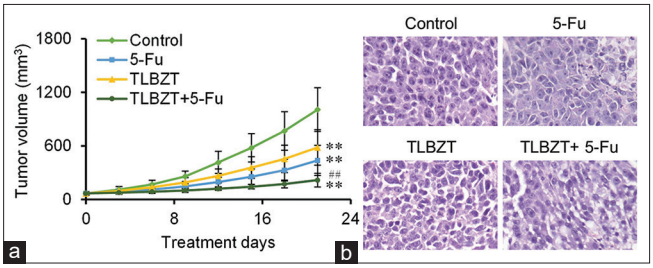

Figure 1: Teng-Long-Bu-Zhong-Tang inhibits tumour growth. (a), Tumour volume. (b), Representative pathological images (H and E, ×200). $^{**}P < 0.01$, versus control group; $^{\#\#}P < 0.01$, versus 5-Fu or Teng-Long-Bu-Zhong-Tang group

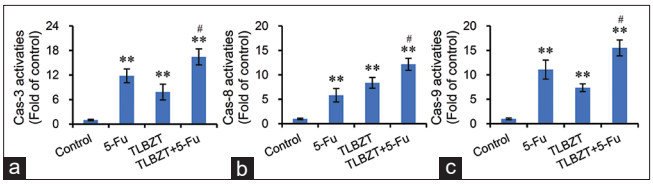

Figure 3: Teng-Long-Bu-Zhong-Tang activates Caspases. Tumours were subjected to Caspase-3 (a), -8 (b), and -9 (c) activities detection. $^{**}P < 0.01$, versus control group; $^{\#}P < 0.05$, versus 5-Fu or Teng-Long-Bu-Zhong-Tang group

Teng-Long-Bu-Zhong-Tang inhibited vascular endothelial growth factor A and HIF-1α expression

VEGFA is one of the most potent angiogenic inducers and may be regulated by HIF-1α, a hypoxia sensor.[19] In the present study, VEGFA and HIF-1α expression was detected by IHC. IHC results revealed that TLBZT reduced HIF-1α and VEGFA expression ($P < 0.01$) [Figure 7].

Discussion

TLBZT is composed of Chinese herbs. The root of *Actinidia chinensis* Planch inhibits the proliferation of cancer cells and induces their apoptosis and cell cycle arrest.[20-22] *S. nigrum* L. or its derivatives arrest the cell cycle, elicit apoptosis and autophagy, and enhance the cytotoxicity of chemotherapeutic drugs in CRC cells.[23-25] Treatment with the seed of *Coix lacryma-jobi* L. inhibits cell migration, cell invasion, cell adhesion, and tumor growth in CRC.[26,27] *S. barbata* D. Don elicits apoptosis and inhibits proliferation, invasion, and migration in CRC cells.[28-30] In the present study, we observed that TLBZT inhibited RKO CRC tumor growth.

Apoptosis, also known as programmed cell death, is mediated by caspases, which mainly involve the extrinsic (death receptor) and intrinsic (mitochondrial) pathways.[12,14] In

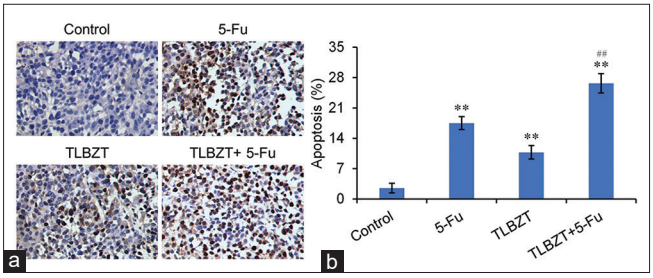

Figure 2: Teng-Long-Bu-Zhong-Tang elicits apoptosis. Apoptosis were evaluated by TdT-mediated dUTP nick end labeling assay, observed under a microscope (×200) (a), analysed by Image-Pro Plus 6.0 software (b). $^{**}P < 0.01$, versus control group; $^{\#\#}P < 0.01$, versus 5-Fu or Teng-Long-Bu-Zhong-Tang group

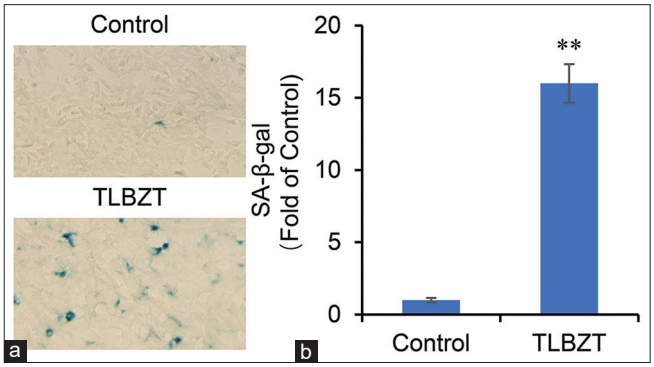

Figure 4: Teng-Long-Bu-Zhong-Tang induces cell senescence. Cell senescence were detected by senescence β-galactosidase staining, observed under a microscope (×200) (a), and analysed by Image-Pro Plus 6.0 software (b). $^{**}P < 0.01$, versus control group

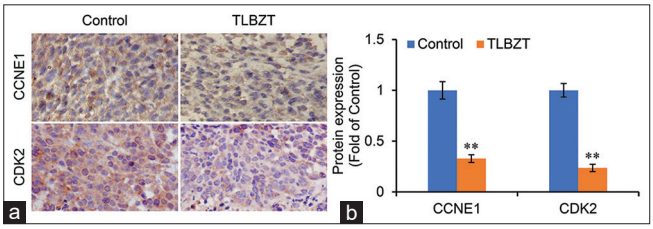

Figure 5: Teng-Long-Bu-Zhong-Tang down-regulates expression of CCNE1 and CDK2. (a), Tumours were subjected to immunohistochemistry with antibodies against CCNE1 and CDK2, observed under a microscope (×200) (a), and quantified by Image-Pro Plus 6.0 software (b). $^{**}P < 0.01$, versus control group

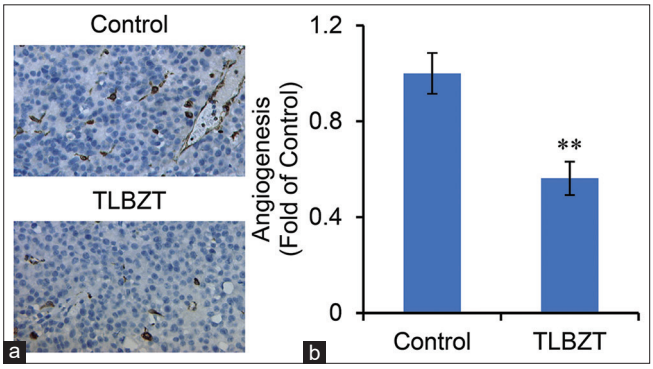

Figure 6: Teng-Long-Bu-Zhong-Tang inhibits angiogenesis. Tumours were subjected to immunohistochemistry with CD31 antibody, observed under a microscope (×200) (a), and analysed by Image-Pro Plus 6.0 software (b). $^{**}P < 0.01$, versus control group

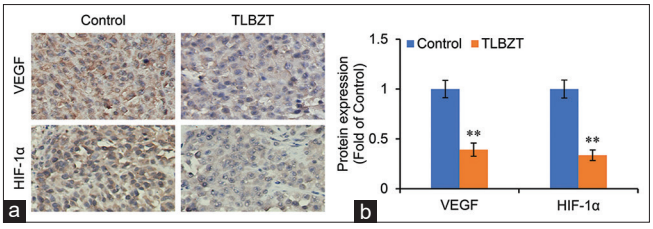

Figure 7: Teng-Long-Bu-Zhong-Tang inhibits expression of VEGFA and HIF-1α. Tumours were subjected to immunohistochemistry with antibodies against VEGFA and HIF-1α, observed under a microscope (×200) (a), and quantified by Image-Pro Plus 6.0 software (b). $^{**}P < 0.01$, versus control group

the extrinsic pathway, FASLG, TRAIL, or tumor necrosis factor-alpha binds to the corresponding death receptor, which in turn activates caspase-8 and -3 and initiates apoptosis. In the intrinsic pathway, DNA damage or cytotoxic stimuli promote cytochrome c release and activate caspase-9 and-3 to initiate apoptosis. In the present study, TLBZT promoted apoptosis and activated caspase-3, -8, and -9, suggesting that TLBZT-induced apoptosis through both the extrinsic and intrinsic pathways.

Cell senescence involves the irreversible withdrawal of cells from the cell cycle and is closely related to CDKN1a/CDKN2a-RB signal transduction.[13,14] CDKN1a and CDKN2a inhibit RB phosphorylation. Hypo-phosphorylated RB binds to E2F and inhibits the transcription of its target genes, such as *CCNE1* and *CDK2*, resulting in G0/G1 phase cell cycle arrest and cell senescence. In the present study, SA-β-Gal staining was used to identify cell senescence. The results showed that TLBZT promoted RKO CRC cell senescence and inhibited CCNE1 and CDK2 expression, suggesting that TLBZT-induced cell senescence was mediated by the expression of CCNE1 and CDK2.

Angiogenesis is the basis for tumor growth and metastasis. Angiogenesis-targeted tyrosine kinase inhibitors, such as aflibercept, and antibodies, such as ramucirumab and bevacizumab, have been approved for CRC treatment.[31,32] VEGFA is a key cytokine that promotes angiogenesis and endothelial cell proliferation, migration, and differentiation.[33] VEGFA expression is regulated by various factors, including HIF-1α. Tumor tissue tends to be hypoxic due to uncontrolled growth, which leads to HIF-1α overexpression that results in a poor prognosis of CRC.[34] HIF-1α can induce the transcription of target genes, including *VEGFA*. TLBZT inhibited angiogenesis and reduced the expression of VEGFA and HIF-1α, suggesting that its effects on angiogenesis could be related to HIF-1α/VEGFA signaling.

Conclusions

In summary, TLBZT inhibited RKO CRC growth, activated caspase-3, -8, and -9 to elicit apoptosis, inhibited CDK2 and CCNE1 expression to promote cell senescence, and inhibited angiogenesis by downregulating HIF-1α/VEGFA signaling. TLBZT also enhanced the anti-cancer effects of 5-Fu on RKO CRC. The present and previous studies suggest that TLBZT is an effective herbal formulation for the inhibition of CRC growth and metastasis.

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Conflicts of interest

There are no conflicts of interest.

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