

Original Article

Tubeimoside-1 inhibits the growth and invasion of colorectal cancer cells through the Wnt/ β -catenin signaling pathway

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Abstract: Tubeimoside-1 (TBMS1) is considered to have anti-tumor properties. However, the role of TBMS1 on human colorectal cancer (CRC) is still unclear. Therefore, in this study, we investigated the role of TBMS1 on human CRC and explored the underlying mechanism. The cell proliferation of CRC cells was detected by MTT assay. Cell migration and invasion were assessed by Boyden chamber assay, and the involvement of molecular mechanisms was examined by western blot. In this study, we found that TBMS1 inhibited the proliferation, migration/invasion of CRC cells, and it reduced β -catenin expression in CRC cells. Furthermore, overexpression of β -catenin rescued TBMS1-induced proliferation and invasion inhibition, and knockdown of β -catenin potentiated TBMS1-induced proliferation and invasion inhibition. Taken together, our results demonstrate that TBMS1 inhibited CRC cell proliferation and invasion via suppressing the Wnt/ β -catenin signaling pathway. Therefore, TBMS1 may represent a chemopreventive and/or therapeutic agent in the prevention of CRC.

Keywords: Tubeimoside-1, colorectal cancer, proliferation, invasion, Wnt/ β -catenin signaling pathway

Introduction

Colorectal cancer (CRC) is the second most cause of cancer-related death [1]. Despite very aggressive treatment including surgery and combined radio and chemotherapy, most patients are initially diagnosed at the advanced stages, there is no effective therapeutic treatment, resulting in short survival time and poor prognosis [2]. Therefore, it is necessary to develop more effective therapeutic agents for CRC.

In recent years, the natural medicine was widely used in clinical and medical research because of its low toxicity and their high biological activity. Tubeimoside-1 (TBMS1), a natural compound isolated from the tuber of Chinese medicinal herb *Bolbostemma paniculatum* (Maxim) Franquet (*Cucurbitaceae*), has sugar chains that are connected with 3-hydroxy-3-methylglutaric acid to form a unique macro-

cyclic structure [3]. It exerts a broad range of important biological actions including anti-inflammatory, antiviral and immunosuppressive effects [4, 5]. In addition, a growing body of evidence indicates that TBMS1 has significant antitumor effects, such as inhibit the cell proliferation, arrest the cell cycle, and promote apoptosis of the human liver [6], squamous esophageal carcinoma [7], choriocarcinoma [8] or lung cancer cells [9].

However, the role of TBMS1 on human CRC is still unclear. Therefore, in this study, we investigated the role of TBMS1 on human CRC and explored the underlying mechanism.

Materials and methods

Materials

TBMS1 was purchased from National Institute for the Control of Pharmaceutical and Biological

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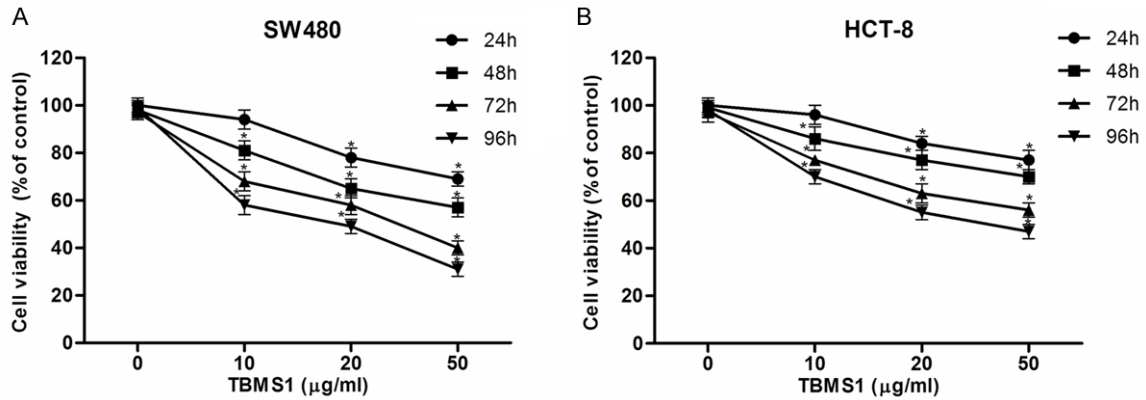


Figure 1. Tubeimoside-1 (TBMS1) inhibits CRC cell proliferation. CRC cells were treated with various concentrations of TBMS1 (0, 10, 20 and 50 µg/ml) for 24 h, 48 h, 72 h and 96 h, and the cell proliferation was determined using MTT assay. TBMS1 inhibited proliferation of SW480 (A) and HCT-8 cells (B) in a time- and concentration-dependent manner, respectively. All experiments were repeated at least three times. *P<0.05 vs control group.

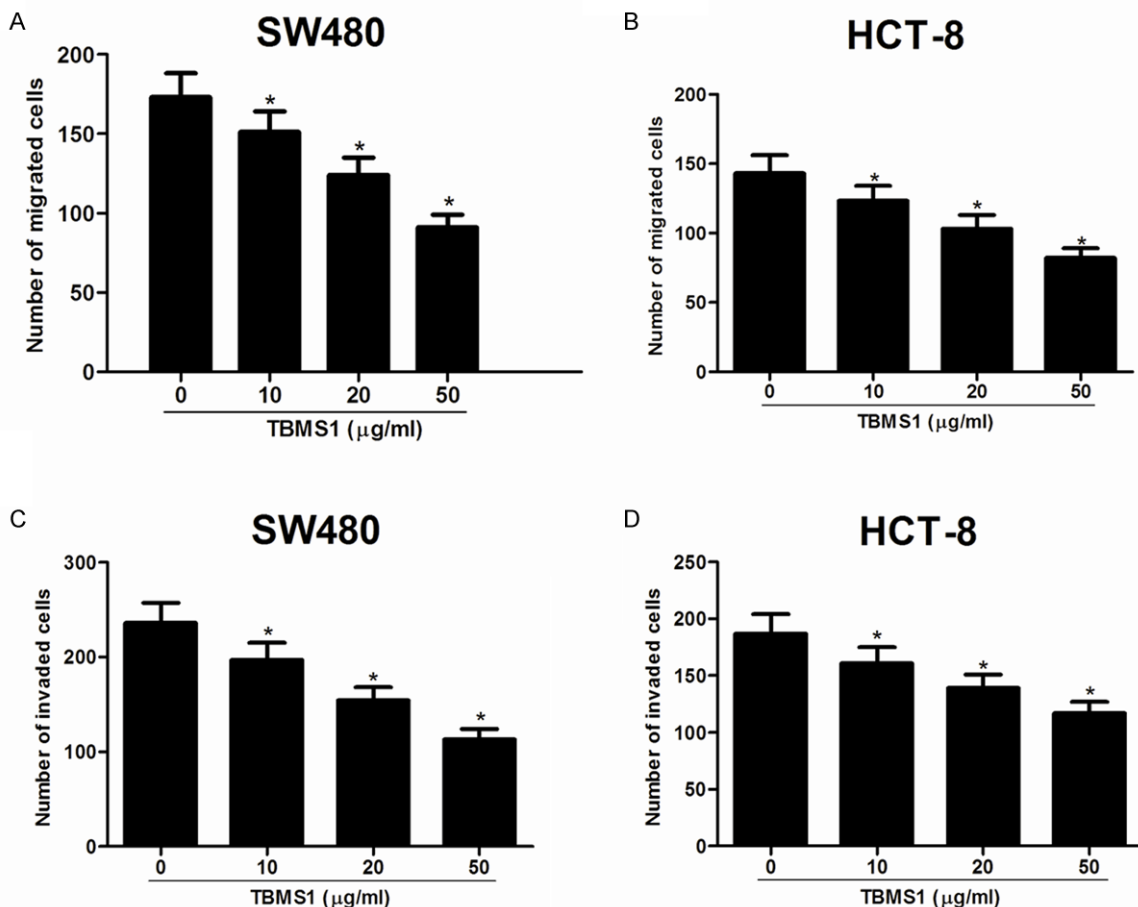


Figure 2. TBMS1 inhibits CRC cell migration and invasion. Migration was determined using Boyden chambers without Matrigel. Invasion was evaluated using Matrigel Boyden chambers. The number of migrating cells was decreased in SW480 (A) and HCT-8 cells (B) treated with TBMS1. The number of invading cells through the Matrigel-coated pores was also reduced in SW480 (C) and HCT-8 cells (D) treated with TBMS1. Data are mean ± SD. All experiments were repeated at least three times. *P<0.05 vs control group.

Products (China), dissolved in phosphate buffered saline (PBS) and stored at -20°C. All other

chemicals, except otherwise noted, were purchased from Sigma.

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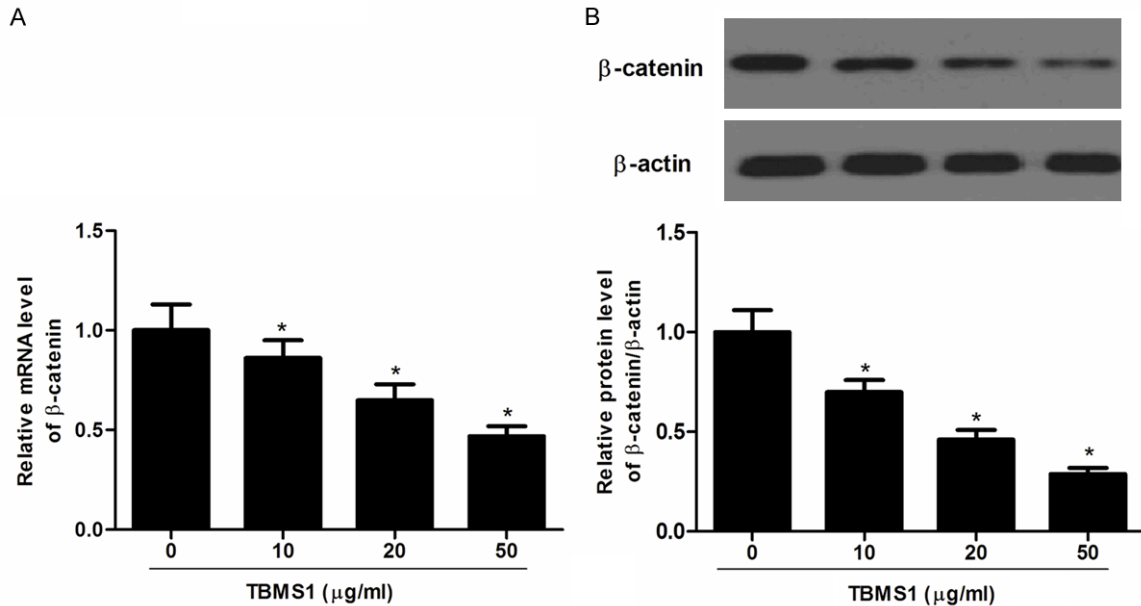


Figure 3. TBMS1 down-regulates the expression of β -catenin in SW480 cells. A. Representative images of relative mRNA level of β -catenin treated with various concentrations of TBMS1; B. Represent western blots of β -catenin. The expression levels of proteins were normalized based on the β -actin levels. Data are mean \pm SD. All experiments were repeated at least three times. * P <0.05 vs control group.

Cell culture

Human CRC SW480 and HCT-8 cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in the RPMI-1640 medium (Gibco, Rockville, MD), with supplements of 10% (v/v) fetal bovine serum (FBS; Gibco, Rockville, MD) and 100 units/ml streptomycin and penicillin (Gibco, Rockville, MD) in a humidified atmosphere containing 5% CO_2 incubator at 37°C.

Cell proliferation assay

Cell proliferation was evaluated by MTT assay. Cells were suspended at 1×10^4 cells/well and 200 μ l of suspension was plated onto each well of a 96-well plate. After 24 h, the medium was replaced by various concentrations of TBMS1 (0, 10, 20 and 50 μ g/ml). At the end of treatment, the medium was removed, and 20 μ l 5 mg/ml MTT in DMEM medium was added. The cells were further incubated in 5% CO_2 at 37°C for 4 h. Formazan was solubilized with 100 μ l dimethylsulfoxide (DMSO) for 10 min. The absorbance (OD) was measured with a microplate reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 570 nm.

Cell migration and invasion assay

The migration and invasion assays were performed as in a 24-well Boyden chamber with 8 μ m pore size polycarbonate membrane (Millipore, Boston, MA, USA). For migration assay, 200 μ l of serum-free medium was added to the upper compartment of the chamber, and 750 μ l RPMI 1640 with 10% FBS was added into the lower compartment. After incubation at 37°C for 24 h, the tumor cells remaining inside the upper chamber were removed with cotton swabs. The cells on the lower surface of the membrane were fixed in 95% ethanol and stained with 0.1% crystal violet. The number of migrated cell was counted under a light microscope. The invasion assay was done by the same procedure, except that the membrane was coated with Matrigel to form a matrix barrier. The experiments were performed in triplicate.

Quantitative real-time-PCR (qRT-PCR) assay

Total RNA was extracted using Trizol Reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Then, 2 μ g of total RNA was transcribed to first-strand cDNA

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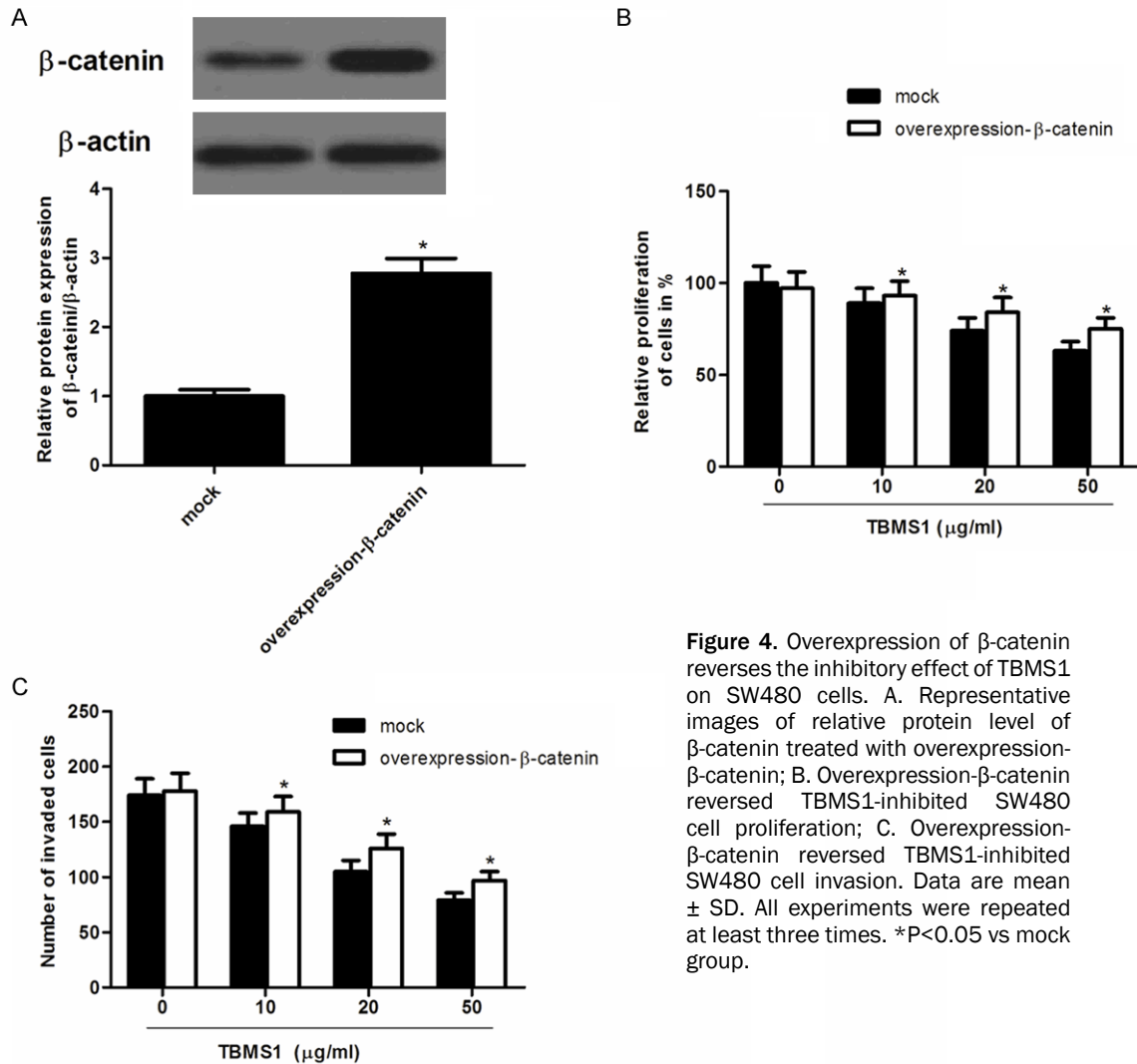


Figure 4. Overexpression of β -catenin reverses the inhibitory effect of TBMS1 on SW480 cells. A. Representative images of relative protein level of β -catenin treated with overexpression- β -catenin; B. Overexpression- β -catenin reversed TBMS1-inhibited SW480 cell proliferation; C. Overexpression- β -catenin reversed TBMS1-inhibited SW480 cell invasion. Data are mean \pm SD. All experiments were repeated at least three times. * $P < 0.05$ vs mock group.

using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). The following primers were used: β -catenin, 5'-GAGT-GCTGAAGGTGCTATCTGTCTG-3' (sense), 5'-GTTCTGAAAAGACGTTGACTTGGA-3' (antisense); and β -actin 5'-CCGTGAAAAGATGACCCAGATC-3' (sense), 5'-CACAGCCTGGATGGCTACGT-3' (antisense). Reactions were carried out using the Step One Plus real-time PCR machine (Applied Biosystems, Carlsbad, CA). And the relative expression was calculated with the $2^{-\Delta\Delta CT}$ equation [10].

Western blot

The cells were lysed with lysis buffer containing 0.5% NP-40, 50 mM Tris-Cl (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA), and protease inhibitor cocktail (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The samples were incu-

bated on ice for 20 min and then centrifuged at 20,000 g for 10 min. The supernatants were collected and the protein concentration was determined using a Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). 25 μ g of protein was loaded and separated in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Then, membranes were blocked with 5% fat-free milk, and incubated with primary antibodies (anti- β -catenin or β -actin) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The signals were determined using an enhanced chemiluminescence (Gibco, Rockville, MD), and the anti- β -actin antibody was used as a loading control.

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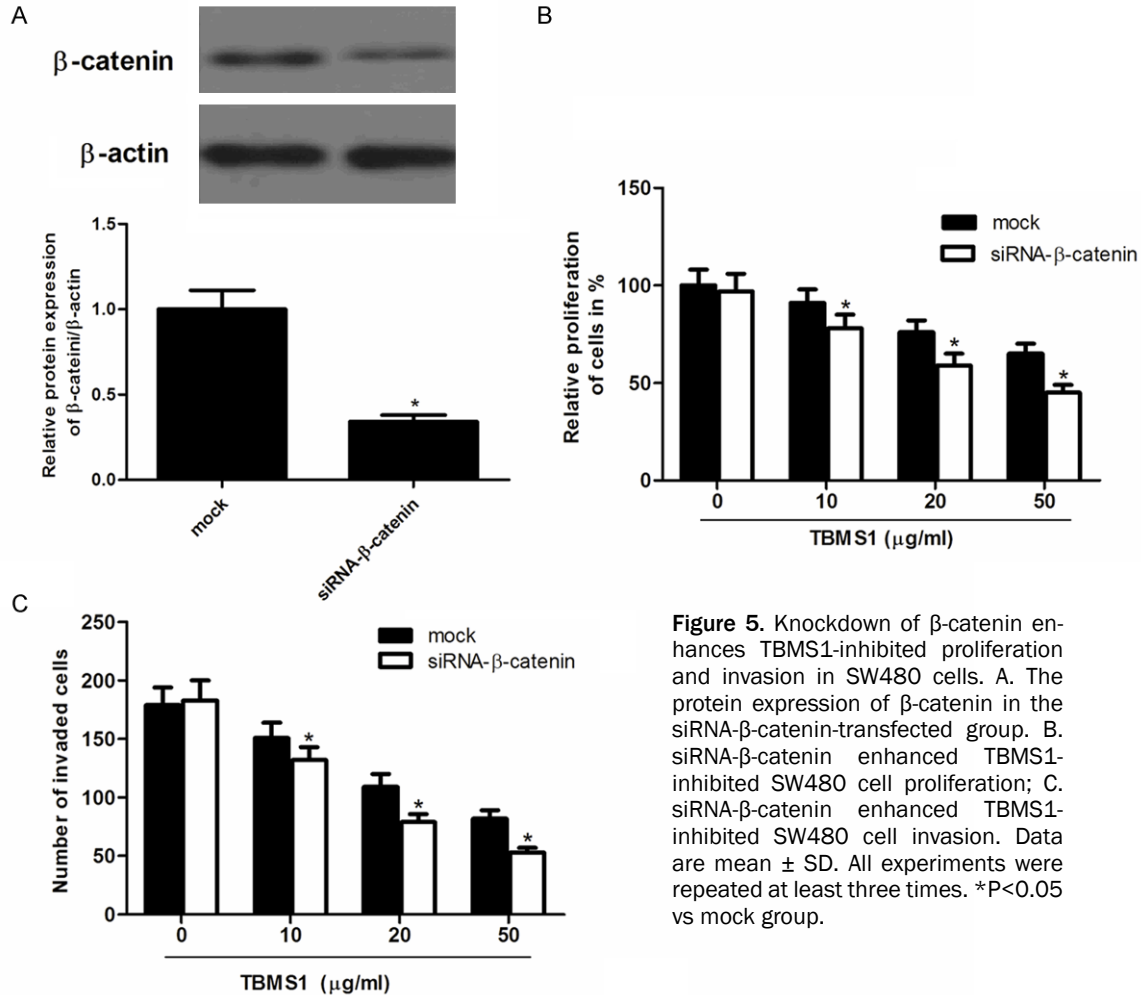


Figure 5. Knockdown of β -catenin enhances TBMS1-inhibited proliferation and invasion in SW480 cells. A. The protein expression of β -catenin in the siRNA- β -catenin-transfected group. B. siRNA- β -catenin enhanced TBMS1-inhibited SW480 cell proliferation; C. siRNA- β -catenin enhanced TBMS1-inhibited SW480 cell invasion. Data are mean \pm SD. All experiments were repeated at least three times. * $P < 0.05$ vs mock group.

Vector construction and transfection

Human full-length β -catenin cDNA was amplified by reverse transcription polymerase chain reaction using mRNA extracted from SW480 cells. Then, the open reading frame of β -catenin cDNA was cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) to generate the recombinant pcDNA3.1- β -catenin expression vector. The small interfering RNA expression vector that expresses β -catenin was purchased from GenePharma Co., Ltd (Shanghai, China). The β -catenin overexpression and siRNA vectors were transfected into SW480 cells using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Results were checked by western blot at 48 h after transfection.

Statistical analysis

Data are expressed as mean \pm SD of triplicate samples. The data significance was evaluated

by using Student's t-test. $P < 0.05$ was considered a statistically significant difference.

Results

TBMS1 inhibits the proliferation of CRC cells

To determine whether TBMS1 affected CRC cell proliferation, MTT assay was performed and results demonstrated that TBMS1 dramatically inhibited proliferation of SW480 cells in a time- and concentration-dependent manner, compared to that of control cells (**Figure 1A**). Similarly, TBMS1 also suppressed the proliferation of HCT-8 cells (**Figure 1B**).

TBMS1 inhibits the migration and invasion of CRC cells

To determine whether TBMS1 affected cell motility, the transwell without Matrigel migration assay was carried out. As shown in **Figure 2**, the mean number of migrated cells per field

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of view was significantly less in SW480 cells treated with TBMS1 than that in control groups (**Figure 2A**). Likewise, TBMS1 obviously inhibited migration of HCT-8 cells compared to that of control cells (**Figure 2B**). Furthermore, the transwell Matrigel invasion assay also demonstrated that TBMS1 reduced invasion activities of SW480 (**Figure 2C**) and HCT-8 cells (**Figure 2D**).

TBMS1 reduced β -catenin expression in CRC cells

The Wnt/ β -catenin signaling pathway was reported to play important roles in CRC proliferation and invasion [11-13]. To understand the molecular mechanism involved in TBMS1-induced proliferation inhibition, we investigated the effect of TBMS1 on β -catenin involved in Wnt/ β -catenin signaling pathway. β -catenin expression in SW480 cells treated with increasing concentrations of TBMS1 for 48 h was assessed using western blot. As shown in **Figure 3**, TBMS1 obviously inhibited the expression of β -catenin in SW480 cells.

Overexpression of β -catenin rescued TBMS1-induced proliferation and invasion inhibition

Next, we investigated the effect of β -catenin overexpression on TBMS1-induced proliferation and invasion inhibition. SW480 cells transfected β -catenin overexpression were treated with 15 μ M TBMS1 for 48 h. As shown in **Figure 4A**, β -catenin protein level in SW480 cells transfected with β -catenin overexpression was obviously increased compared with the negative group. In addition, β -catenin overexpression significantly increased the cancer cell proliferation and invasion, and β -catenin overexpression rescued TBMS1-induced cell growth (**Figure 4B**) and invasion inhibition (**Figure 4C**).

Knockdown of β -catenin potentiated TBMS1-induced proliferation and invasion inhibition

To further confirm the role of β -catenin in the proliferation and invasion of CRC cells, we performed a gene knockdown experiment in SW480 cells. As shown in **Figure 5A**, β -catenin protein level in SW480 cells transfected with siRNA- β -catenin was obviously decreased compared with the negative group. In addition, knockdown of β -catenin significantly potentiated TBMS1-induced proliferation and invasion inhibition (**Figure 5B** and **5C**).

Discussion

TBMS1 has been reported to possess anticancer properties. However, the role of TBMS1 on human CRC is still unclear. In this study, we found that TBMS1 inhibited the proliferation, migration/invasion of CRC cells, and it reduced β -catenin expression in CRC cells. Furthermore, overexpression of β -catenin rescued TBMS1-induced proliferation and invasion inhibition, and knockdown of β -catenin potentiated TBMS1-induced proliferation and invasion inhibition.

TBMS1 is a triterpenoid saponin extracted from the tubers of *Bolbostemma paniculatum* (Maxim). Franquet (*Cucurbitaceae*). It has been reported that TBMS1 inhibited the proliferation of the cells in a dose- and time-dependent manner in lung cancer cells [9], and it also inhibited BGC823 gastric cancer cell proliferation in a concentration- and time-dependent manner [14]. Similar studies have been performed with other triterpenoid saponins. For example, Raddeanin A is an active triterpenoid saponin from a traditional Chinese medicinal herb, *Anemone raddeana* Regel, and it inhibits the angiogenesis and growth of human CRC [15]. Protopanaxadiol, an important component of American ginseng, also suppresses cancer cell growth inhibition by promoting G1 cell cycle redistribution and apoptosis in human CRC cell lines [16]. In line with these reports, in this study, we found that TBMS1 inhibited the proliferation, migration/invasion of CRC cells, suggesting that TBMS1 may function to inhibit the development and progression of CRC.

A growing body of evidence demonstrates that the Wnt/ β -catenin signaling pathway plays important roles in the progression of various human cancer types through modulation of many biological processes, including cell growth, invasion and metastasis, apoptosis, differentiation [17-20]. The Wnt-signaling pathway regulates gene expression by stabilizing β -catenin, which translocates to the nucleus and forms complexes with T-cell factor transcription factors [21]. There is ample *in vitro* and *in vivo* evidence that Wnt/ β -catenin signaling is involved in CRC tumorigenesis. For example, it is overexpressed in eight samples of fresh colorectal cancer tissues compared with their respective adjacent non-tumor colorectal

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mucosa tissues [22]. Gao *et al* demonstrated that differential β -catenin expression levels were associated with aggressive morphological features, epithelial-mesenchymal transition and a poor prognosis in CRC [23]. Furthermore, it has been reported that downregulation of β -catenin inhibits invasion and migration of colon cancer cells *in vitro* [24]. Abnormal β -catenin expression also upregulated the expression of cyclinD1, c-Myc and matrix metalloprotease7, leading to uncontrolled cell proliferation and invasion [25]. Consistent with previous studies, in this study, we found that TBMS1 significantly inhibited the expression of β -catenin in SW480 cells. In addition, overexpression of β -catenin rescued TBMS1-induced proliferation and invasion inhibition, and knock-down of β -catenin potentiated TBMS1-induced proliferation and invasion inhibition. All these results suggest that TBMS1 inhibited CRC cell proliferation and invasion via the Wnt/ β -catenin signaling pathway.

In summary, this study demonstrated that TBMS1 inhibited CRC cell proliferation and invasion via suppressing the Wnt/ β -catenin signaling pathway. Therefore, TBMS1 may represent a chemopreventive and/or therapeutic agent in the prevention of CRC.

Acknowledgements

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Disclosure of conflict of interest

None.

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