An ethanolic extract of Bailian (Radix Ampelopsis Japonicae): demonstration of colorectal cancer treatment efficacy via inhibition of β-catenin signaling in vitro

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OBJECTIVE: To investigate the underlying mechanism of Bailian (Radix Ampelopsis Japonicae, BL) extract action on colorectal cancer (CRC).

METHODS: We explored the involvement of β-catenin signaling on the anti-CRC effects of an BL ethanolic extract (BLE) in cell models by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, immunofluorescent staining, luciferase assay, Western blot analysis and real-time quantitative polymerase chain reaction analysis. Anti-CRC compounds were quantified by high performance liquid chromatography.

RESULTS: The contents of gallic acid, catechin, and epicatechin in the BLE were 0.23, 1.25, and 0.18 g/kg, respectively. BLE-mediated cytotoxic and apoptotic effects were accompanied by lowered β-catenin/Tcf transcriptional activity, reduced β-catenin nuclear localization, and downregulated protein and mRNA levels of both β-catenin and molecules regulated by β-catenin.

CONCLUSION: The mechanism underpinning the anti-CRC effects of BLE may involve inhibition of β-catenin signaling. Further studies are necessary to establish the role of β-catenin signaling in the action of BLE-mediated anti-CRC effects.

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Keywords: Bailian (Radix Ampelopsis Japonicae); Colorectal neoplasms; Beta Catenin; Cytotoxins; Apoptosis

INTRODUCTION
Colorectal cancer (CRC) is the third most common...
cause of cancer mortality, and its incidence is rapidly increasing worldwide. Although targeted therapy and immunotherapy are showing promising clinical results, the currently available chemotherapeutics against CRC often have toxicities and low response rates. These highlight the urgency of exploring effective and safe targeted therapeutic agents for CRC management. Because of their biological activity and low toxicity, the largely unexplored, Traditional Chinese Medicine (TCM)-based herbs with multi-component and multi-target nature have advantages in managing complex diseases, including CRC. Aberrant activation of β-catenin is frequently detected in CRC. Moreover, β-catenin signaling has been proposed as a novel target for treating CRC.

Ampelopsis Radix, the dried root tuber of Bailian (Radix Ampelopsis Japonica) (BL), is a low toxicity traditional Chinese medicinal herb commonly prescribed by TCM doctors for treating various cancers including CRC. Some constituents of BL, including gallic acid, catechin, epicatechin, quercetin, and lupeol” have been reported to exert anti-cancer effects by inhibiting β-catenin signaling. However, the anti-CRC mode and mechanism of action of BL are not fully elucidated. Our previous studies demonstrated that an ethanolic extract of BL (BLE) exerts anti-CRC effects and potently inhibits STAT3 signaling. Here, we explored the involvement of β-catenin signaling in the anti-CRC effects of BLE.

MATERIALS AND METHODS

**Chemicals, reagents and herbal materials**

Antibodies against cyclin D1, survivin, c-Myc, cleaved-caspase-3, β-catenin, and anti-rabbit IgG Fab2 Alexa Fluor were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and SP1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-rabbit IgG, goat anti-mouse IgG, and protein marker were provided by Bio-Rad (Hercules, CA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemicals Ltd. (St. Louis, MO, USA). Fetal bovine serum (FBS), Dulbecco’s Modified Eagle Medium (DMEM), penicillin, and streptomycin were obtained from HyClone (Logan, UT, USA). Human CRC HCT-116 and SW480 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All materials for cell culture were obtained from Life Technologies Inc., (GIBCO, Carlsbad, CA, USA). Preparation and quality control of BLE were described previously. The contents of the anti-CRC compounds gallic acid, catechin, and epicatechin in BLE were 0.23, 1.25, and 0.18 g/kg, respectively.

**Cell culture**

HCT-116 and SW480 cells were cultured in medium containing 5% heat inactivated FBS and 1% penicillin/streptomycin at 37 °C in an atmosphere with 5% CO2.

**Cell viability assay**

HCT-116 and SW480 cells were seeded on 96-well plates (5000 cells/well) and allowed to adhere overnight. The cells were treated with various concentrations of BLE as indicated for 24, 48, or 72 h. Then, 20 µL of MTT solution (5 mg/mL) was added to each well and incubated for an additional 3 h. The formazan crystal formed was dissolved with 100 µL of DMSO and absorbance was detected at 570 nm using a microplate spectrophotometer (BD Biosciences, San Jose, CA, USA).

**Immunofluorescent staining**

HCT-116 and SW480 cells were seeded on sterile coverslips in 24-well plates and allowed to grow in serum-free medium for 12 h. After treatment with vehicle or BLE (100 µg/mL) for 48 h, cells were fixed in 3.7% formalin and washed with phosphate buffered saline (PBS). Nonspecific sites were then blocked with PBS containing 5% bovine serum albumin for 30 min at room temperature with gentle rocking. Thereafter, a solution of anti-cleaved-caspase-3 antibody (1: 500) was flooded over the cells, and the cultures were incubated at 4 °C overnight. After washing with PBS, cells were further incubated with anti-rabbit IgG Fab2 Alexa Fluor (Cell Signaling Technology, USA) for 1 h at room temperature, followed by washing with PBS and analysis using a fluorescence microscope (Leica, Germany).

**Luciferase assay**

The TOP (WT Tcf binding site) and FOP (mutated Tcf binding site) luciferase reporters were obtained from Addgene (Cambridge, MA, USA). The plasmid transfection and luciferase assay were performed as described previously. Firefly and renilla luciferase activity were assayed following the manufacturer’s protocol (Promega) (Madison, WI, USA). Firefly luciferase activity in the cell lysate was normalized to that of renilla luciferase and was expressed as an average of three independent experiments.

**Preparation of cytoplasmic and nuclear fractions**

HCT-116 and SW480 cells were seeded on 6-well plates (5 x 10⁴ cells/well) and allowed to adhere overnight. The cells were then treated with vehicle or various concentrations of BLE. After incubation for 24 h, BLE- and vehicle-treated cells were collected and suspended in hypotonic buffer. After incubation on ice for 15 min, 12 µL 10% (v/v) NP-40 was added, and the samples were vortexed for 10 s and kept on ice for another 10 min. After centrifugation at 25 000 g at 4 °C for 1 min, the supernatants (cytoplasmic extracts), were transferred to fresh tubes. The pellets were...
washed once with 100 μL hypotonic buffer [20 mM Tris-HCl (pH 7.9), 1.5 mM MgCl2 and 10 mM KCl], and resuspended in high salt buffer [20 mM Tris-HCl pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl2 and 0.2 mM EDTA]. After 30 min incubation on ice, the lysates were centrifuged at 25 000 g at 4 °C for 10 min and the supernatants were kept as nuclear extracts.²⁴

**Western blot analysis**

HCT-116 and SW480 cells were seeded and treated as described in the “Preparation of cytoplasmic and nuclear fractions” section above. Cell extracts were prepared, and electrophoresed under denaturing conditions.³⁶ The proteins were transferred onto polyvinylidifluoridine membranes. The membranes were washed in tris buffered saline containing 0.05% (v/v) Tween-20 and incubated over night at 4°C with primary antibodies (β-catenin (1 : 1000), GAPDH (1 : 4000), Cyclin D1 (1 : 2000), Survivin (1 : 2000), c-Myc (1 : 1000)). The membranes were then incubated with secondary antibodies and signals were detected using ECL detection reagents (Amersham Biosciences, Piscataway, NJ, USA).²⁵

**Real-time quantitative polymerase chain reaction analysis**

Total RNA was extracted from cells using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was reverse-transcribed into cDNA using PrimeScript™ RT reagent Kit (Takara, Japan). Quantitative real time polymerase chain reaction analyses (PCR) was performed in triplicate using iTaq™ Universal SYBR green polymerase chain reaction systems (Bio-Rad, Hercules, CA, USA) with a Viia 7 Real Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Each sample was amplified in triplicate for quantification. Data were analyzed by relative quantitation using the 2^ΔΔCt method and expression was normalized to that of GAPDH).²⁰

**Statistical analysis**

Data are presented as mean ± standard deviation (x±s). Data were analyzed by one-way analysis of variance followed by Dunnett’s multiple comparisons. Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). P < 0.05 was considered statistically significant.

**RESULTS**

**BLE reduces viability and induces apoptosis in CRC cell**

Consistent with our previous observations, BLE reduced cell viability of HCT-116 and SW480 in both time- and dose-dependent manners (Figure 1).²⁰ Substantial induction of caspase-3 cleavage in BLE-treated CRC cells confirmed the apoptotic effect of BLE (Figure 2).²⁰

**BLE inhibits β-catenin/Tcf transcriptional activity and reduces β-catenin nuclear localization in CRC cells**

We evaluated the effect of BLE treatment on β-catenin/Tcf transcriptional activity by transiently transfecting CRC cells with reporter plasmids with wild type (TOP) or mutated (FOP) Tcf binding sequences. Upon BLE treatment, we observed a dose-dependent decrease in β-catenin/Tcf transcriptional activity (TOP-Flash luciferase assay) in HCT-116 and SW480 cells (Figure 3). We investigated whether this observation was related to a reduction in β-catenin nuclear localization. Western blotting results showed that the levels of β-catenin in nuclear fractions were significantly reduced by BLE in a dose-dependent manner (P < 0.01) (Figure 4).

**BLE decreases β-catenin protein and mRNA levels in CRC cells**

BLE reduced β-catenin protein levels in CRC cells (Figure 5). Most CRC cells have mutations of the adenomatous polyposis coli (APC) gene or the β-catenin gene that stabilize β-catenin, ultimately leading to tumorigenesis.³⁰,³¹ In this study, we used the APC mutated SW480 cells and β-catenin mutated HCT-116 cells. We found that β-catenin protein level was lowered by BLE in both cell lines. Therefore, we hypothesize that the BLE-induced decrease of β-catenin protein levels may directly result from a reduction in β-catenin mRNA levels. BLE dose-dependently lowered mRNA level of β-catenin (Figure 6).

**BLE inhibits the expression of β-catenin/Tcf downstream genes in CRC cells**

Since BLE decreases β-catenin mRNA levels, BLE is anticipated to have an inhibitory effect on β-catenin function. BLE remarkably decreased mRNA and protein levels of the β-catenin/Tcf transcriptional targets survivin, c-Myc, and cyclin D1 in both CRC cell lines (Figures 7 and 8).

**DISCUSSION**

In Shen Nong Ben Cao Jing written 2000 years ago, BL was documented to treat various TCM symptoms that would now be diagnosed as cancers, including CRC. In modern clinical Chinese medicine practice, BL is also used for treating CRC.⁶⁻¹² Our previous¹⁰ and current studies show that BLE has anti-CRC effects in cell models. Our findings, together with the ancient records, suggest that BLE is a safe and effective treatment for CRC. Activated β-catenin signaling, through the accumulation of β-catenin in cells, has been implicated in human carcinogenesis. This accumulation may result from mutation of β-catenin or APC. APC and β-catenin are mutated in many cancer types including...
In this study, we found that inhibition of β-catenin involved in the anti-CRC action of BLE. Some constituents in BLE, including the ones quantified in this study, exhibit anti-CRC effects and mRNA levels (Figures 5, 6). Survivin, c-Myc, and cyclin D1 are β-catenin transcriptional target genes involved in CRC cell survival. In this study, we show that BLE dose-dependently decreases β-catenin protein and mRNA levels (Figures 5, 6). Survivin, c-Myc, and cyclin D1 are β-catenin transcriptional target genes involved in CRC cell survival. Therefore, targeting β-catenin is a useful strategy for developing novel anti-CRC targeted therapies. Some constituents in BLE, including the ones quantified in this study, exhibit anti-CRC effects in part by inhibiting β-catenin signaling. In this study, we show that BLE dose-dependently decreases β-catenin protein and mRNA levels (Figures 5, 6). Survivin, c-Myc, and cyclin D1 are β-catenin transcriptional target genes involved in CRC cell survival. We also showed that BLE treatment dose dependently down-regulates Survivin, c-Myc, and cyclin D1 protein and mRNA levels (Figures 7, 8). Our results suggest that β-catenin is involved in the anti-CRC action of BLE.

Our previous studies showed that BLE exerts anti-CRC effects and inhibits STAT3 signaling in vitro. In this study, we found that inhibition of β-catenin sig-
β-catenin nuclear localization, and downregulation of β-catenin reduces apoptosis. In summary, consistent with the results of our previous studies, we found that BLE reduces cell viability and induces apoptosis. In addition, we demonstrated that BLE reduces β-catenin/Tcf transcriptional activity, decreases β-catenin nuclear localization, and downregulates mRNA and protein levels of β-catenin and molecules regulated by β-catenin. We conclude that inhibiting β-catenin signaling is associated with the anti-CRC effects of BLE. Further studies are warranted to establish the role of β-catenin signaling in the anti-CRC effects of BLE and to identify potent anti-CRC compounds in BLE.

Figure 4 BLE reduces β-catenin nuclear localization in CRC cells
A: representative immunoblotting images in HCT-116 cells; B: relative protein levels in HCT-116 cells; C: representative immunoblotting images in SW480 cells; D: relative protein levels in SW480 cells. Cells were treated with the indicated concentrations of BLE or vehicle for 24 h. Levels of β-catenin protein were examined in nuclear extracts by immunoblotting and relative (to SP-1) levels were analyzed by Image J software (right). Data were presented as the mean ± standard deviation of three independent experiments. BLE: Bailian (Radix Ampelopsis Japonicae) extract; CRC: colorectal cancer. *P < 0.01 vs vehicle of each cell line.

Figure 5 BLE decreases β-catenin protein levels
A: representative immunoblotting images in HCT-116 cells; B: relative protein levels in HCT-116 cells; C: representative immunoblotting images in SW480 cells; D: relative protein levels in SW480 cells. β-catenin in CRC cells treated with various concentrations of BLE or vehicle for 24 h was detected by Western blotting. The amount of β-catenin, relative to glyceraldehyde-3-phosphate dehydrogenase, was analyzed by Image J software. Data were presented as the mean ± standard deviation of three independent experiments. BLE: Bailian (Radix Ampelopsis Japonicae) extract; CRC: colorectal cancer. *P < 0.01, #P < 0.05 vs vehicle.

Figure 6 BLE reduces β-catenin mRNA levels
A: relative mRNA level in HCT-116 cells; B: relative mRNA level in SW480 cells. CRC cells were treated with various concentrations of BLE or vehicle for 12 h, total mRNA was extracted for detection of the β-catenin mRNA levels using real-time polymerase chain reaction analyses. Data were presented as the mean ± standard deviation of three independent experiments. BLE: Bailian (Radix Ampelopsis Japonicae) extract; CRC: colorectal cancer. *P < 0.01 vs vehicle of each cell line.
Figure 7 BLE downregulates mRNA levels of β-catenin/Tcf target genes
A: relative mRNA level in HCT-116 cells; B: relative mRNA level in SW480 cells. CRC cells were treated with various concentrations of BLE or vehicle for 12 h, total mRNA was extracted for detection of cyclin D1, survivin, and c-Myc mRNA levels using real-time polymerase chain reaction analyses. All data were presented as mean ± standard deviation from three independent experiments. BLE: Bailian (Radix Angelicae Japonicae) extract; CRC: colorectal cancer. *p < 0.01 vs vehicle (cyclin D1) of each cell line, **p < 0.01 vs vehicle (survivin) of each cell line, ***p < 0.01, ****p < 0.05 vs vehicle (c-Myc) of each cell line.

Figure 8 BLE downregulates the protein levels of β-catenin/Tcf target genes
A: representative immunoblotting images in HCT-116 cells; B: relative protein levels in HCT-116 cells; C: representative immunoblotting images in SW480 cells; D: relative protein levels in SW480 cells. After BLE treatment for 24 h, total cell lysates were extract for Western blot analyses using antibodies specific for cyclin D1, survivin and c-Myc. The representative results and the relative levels as analysed by Image J software were shown. All data were presented as mean ± standard deviation from three independent experiments. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control for determination of relative expression. BLE: Bailian (Radix Angelicae Japonicae) extract; CRC: colorectal cancer. *p < 0.05, **p < 0.01 C vehicle (cyclin D1) of each cell line, **p < 0.05; ***p < 0.01 vs vehicle (survivin) of each cell line, ****p < 0.05, *****p < 0.01 vs vehicle (c-Myc) of each cell line.

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